Mechanism of cell death in Burkitt lymphomas: apoptosis or mitotic catastrophe

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Dedicated

to my family

ZUSAMMENFASSUNG

Bei dem Burkitt-Lymphom handelt es sich um ein hochmalignes Non-Hodgkin-Lymphom, das sich aus reifen B-Lymphozyten entwickelt. Typischerweise ist es mit einer Epstein-Barr-Virusinfektion oder einer chromosomalen Translokation assoziiert, welches zu einer deregulierten Aktivierung des c-myc Onkogens führt. Zur Behandlung wird eine Kombinationstherapie mit Vincristin verwendet, auf die jedoch nur rund 50% der Patienten ansprechen. Ursache hierfür könnte die genetische Instabilität des Tumors sein, die in der Selektion resistenter Subklone und somit zur Entwicklung von Therapieresistenz führt. Apoptoseresistenz wird hierbei als Hauptfaktor der Resistenzbildung beim Burkitt Lymphom angesehen. Um die molekularen Mechanismen der Apoptoseresistenz aufzuklären, wurde die Apoptoseinduktion in 15 Burkitt-Lymphom-Zelllinien nach Behandlung mit den Spindelgiften Taxol (Paclitaxel), Nocodazol und Vincristin untersucht. Interessanterweise entwickeln Zellen, die sich als resistent gegenüber Taxol- und Nocodazol-induzierter Apoptose erwiesen, eine Polyploidie (>4N DNA), was eine inverse Relation von Apoptose und Polyploidie nach Taxol und Nocodazol Behandlung aufzeigt. Die zugrunde liegenden Mechanismen wurden in drei resistenten und vier sensitiven Zelllinien näher charakterisiert. In den sensitiven Zelllinien war die Taxol -und Nocodazol-induzierte Apoptose von Caspase-Aktivierung, Bid-Spaltung und Herunterregulation von Mcl-1 begleitet. Im Gegensatz dazu wiesen die meisten apoptoseresistenten Zellen einen Verlust von Bax und Bak auf und waren durch einen anhaltenden mitotischen Arrest mit Auftreten eines >4N DNA-Gehalts nach Behandlung charakterisiert. Die Inhibierung der Apoptose mittels Caspase-Inhibitoren führte in apoptosesensitiven Zelllinien zum Auftreten von Polyploidie und anschließendem Zelltod durch mitotische Katastrophe, was den inversen Zusammenhang von Apoptose und Polyploidie weiter bestätigt.

Um weitere Einblick in den Mechanismus der Spindelgift-induzierten Apoptose zu erhalten, wurde die Rolle der mitotische Kinase PLK1 (polo-like kinase) näher untersucht. PLK1 hat eine Reihe von Funktionen während des Zellzyklus und ist in ca. 80 % der humanen Tumoren verschiedensten Ursprungs überexprimiert. Erhöhte PLK1 Expression korreliert bei vielen Tumorarten mit einer schlechten Prognose, so auch bei Non-Hodgkin-Lymphomen. In dieser Studie konnten wir zeigen, dass Verlust der PLK-1 Funktion in Burkitt-Lymphomen die Zellzyklus-Transitionskontrolle beim Eintritt der Zelle aus der G2-Phase in die Mitose

aktiviert. Hemmung von PLK1 interferiert allerdings nicht mit der Apoptose-Induktion durch Spindelgifte, was darauf hindeutet, dass der ausgelöste Zelltod durch Mikrotubuli-destabilisierende Agenzien keinen geordneten Ablauf der Mitose voraussetzt. Des Weiteren induzierte eine dominant-negative PLK1-Mutante Apoptose. Die zusätzliche Behandlung mit Spindelgiften zeigte hier keinen synergistischen Effekt, was darauf schließen lässt, dass sowohl Inhibierung von PLK1 als auch Mikrotubuli-destabilisierende Agenzien den gleichen Stress-Signalweg aktivieren. Tatsächlich führt die Hemmung der Caspaseaktivität in Burkitt-Lymphomen und in der Zervixkarzinom-Zelllinie HeLa zur Polyploidie. Andererseits unterstützt Überexpression von Wildtyp-PLK1 in Taxol behandelten Zellen die Zellzyklus-Progression. Dies deutet auf eine Verbindung zwischen Zelltodresistenz und genetischer Instabilität (Aneuplodie) hin.

Anders als Taxol und Nocodazole induziert Vincristine keine Polyploidie. Da sich die Entwicklung von polyploiden Tumorzellen aus der chromosomalen Instabilität als Folge der Störung der mitotischen Spindel darstellt, kann dies als kritisches Ereignis bei der Entstehung von Tumorzellen angesehen werden. Die Tatsache, dass Vincristin nicht in der Lage ist, ein solches unerwünschtes Ereignis zu induzieren, könnte erklären warum sich Vincristin besser zur Behandlung von Lymphomen eignet als Taxane. Medikamente, welche die Caspase-Aktivierung unabhängig von Bax und Bak induzieren, könnten eine weitere Möglichkeit zur Behandlung von resistenten Burkitt-Lymphomen darstellen.

ABSTRACT

Burkitt lymphoma is a fast-growing Non-Hodgkin's Lymphoma that originates from mature B cells. It is generally associated with chromosomal translocations or/and Epstein–Barr virus infection leading to deregulated activation of the c-myc oncogene. Combination chemotherapy with a vincristine-containing regimen is the treatment of choice in Burkitt lymphoma, however, only 50% of the patients respond to treatment due to the high genetic instability, selection of resistant tumor cell subclones and development of clinical resistance to therapy. Apoptosis resistance is considered as the major cause of resistance in Burkitt lymphomas. To elucidate molecular abnormalities that are responsible for resistance, 15 Burkitt lymphoma cell lines were investigated for apoptosis induction upon treatment with the microtubule inhibitors taxol (paclitaxel), nocodazole and vincristine. Interestingly, cell lines, which are highly resistant to apoptosis induction showed development of polyploidy (>4N cellular DNA content) and vice versa, displaying an inverse relationship between apoptosis and polyploidy induction upon treatment with taxol or nocodazole. The underlying mechanism was characterized in three resistant and four sensitive prototypic cell lines. In sensitive cell lines, taxol- and nocodazole-induced apoptosis was accompanied by caspase activation, Bid cleavage and Mcl-1 downregulation. In contrast, most apoptotic resistant cell lines exhibited a loss of Bax/Bak and showed prolonged mitotic arrest with >4N DNA content upon treatment. Interestingly, inhibition of apoptosis in sensitive cells by caspase inhibition promoted polyploidy and subsequent death by a mitotic catastrophe confirming the inverse relationship between apoptosis and polyploidisation. To gain mechanistic insights into microtubule inhibitor-induced cell death, the role of the mitotic kinase PLK1 was addressed. PLK1 has multiple functions during the cell cycle and is known to be overexpressed in 80% of human tumors of diverse origins. Elevated levels of PLK1 correlate with poor prognosis for a wide range of human cancers, including Non-Hodgkin Lymphoma. Here, abrogation of PLK1 function in Burkitt lymphomas induces cell cycle checkpoint activation at G2 and M phase. Blocking the function of PLK1, however, did not interfere with cell death induced by the spindle toxins indicating that cell death induced by microtubule disrupting agents does not require coordinated transition through mitosis. Moreover, a dominant negative PLK1 mutant induced apoptosis. Additional treatment with microtubule inhibitors failed to show synergism in induction of apoptosis indicating that PLK1 inhibition and spindle toxins might

trigger a similar mitotic stress pathway. Infact, lack of caspase activity promotes polyploidy in Burkitt lymphomas and cervical carinoma (HeLa cells). Conversely, overexpression of wildtype PLK1 promoted cell cycle progression in cells treated with taxol. This indicates a link between cell death resistance and genomic instability (aneuploidy) of Burkitt lymphomas. Unlike taxol or nocodazole, vincristine treatment failed to induce polyploidy. Since polyploidy development of tumor cells occurs in consequence of chromosomal imbalances following disruption of the mitotic spindle machinery this might be a critical event leading to chromosomal imbalances in tumor cells. The failure of vincristine to induce such an undesirable event may explain why vincristine is a better treatment option as compared to taxanes in lymphoma therapy. Furthermore, drugs inducing caspase activation independently of Bax and Bak deficiency might represent an option to treat resistant Burkitt lymphomas.

1 INTRODUCTION

1.1 Cell death

Cell death is a highly controlled event, which plays an essential role in developmental biology and tissue homeostasis. Apart from biochemical processes taking place in the dying cell, execution ultimately also involves the orderly removal of dying cells and cellular debris. Processes of cell death and cell proliferation act to keep appropriate cell numbers or eliminate cells that are functionally redundant or potentially detrimental to the organism (Ullrich, et al., 2008). For instance induction of cell death is important for the removal of cells when there is organelle dysfunction (mitochondria, endoplasmic reticulum), DNA damage, abnormal protein folding/accumulation or oncogene activation when cells are exposed to harmful viral infection (Twomey and McCarthy, 2005).

Table 1: Cell death-some historical aspects. Scientific discoveries, which made great impact on the development of the very field of cell death

Year	Scientist name	Discovery
1858	Rudolf Virchow	necrosis: degeneration, softening, advanced tissue breakdown (i.e., gangrene)
1885	Walter Flemming	spontaneous cell death, “chromatolysis” in ovarian follicles
1886	Nissen	Chromatolysis in lactating mammary glands
1910	Von Recklinghausen	Proposed the therm oncosis (swelling precisely to mean cell death with swelling)
1955	Christian de Duve	discovery of lysosomes, concept of cell suicide
1964	Lockshin and Williams	“programmed cell death”
1971	Kerr, Wyllie and Currie	apoptosis: apo= away from, ptosis= falling
1984	Wyllie et al.	Demonstrated discrete inter-nucleosomal DNA fragmentation in apoptosis (as a biochemical marker for apoptosis)

Cell death is classified according to its morphological appearance (which may be apoptotic (Type I cell death), necrotic, autophagic (Type II cell death) or associated with mitosis i.e mitotic catastrophe), enzymological criteria (with and without the involvement of nucleases or of distinct classes of proteases, such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics (immunogenic or non-immunogenic).

According to the NCCD (The Nomenclature Committee on Cell Death), cell death is characterized if one of the following molecular or morphological criteria is met: (1) the cell has lost the integrity of its plasma membrane, as defined by the incorporation of vital dyes [e.g., Propidium Iodide (PI)] *in vitro*; (2) the cell, including its nucleus, has undergone complete fragmentation into discrete bodies (which are frequently referred to as 'apoptotic bodies'); and/or (3) its corpse (or its fragments) has been engulfed by an adjacent cell *in vivo*. In particular, cells that are arrested in the cell cycle, which alludes to the loss of clonogenic potential, are considered as alive (Kroemer, et al., 2005). Pathological conditions, such as cancer and autoimmunity, are very often associated with deregulation of apoptosis signalling pathways and defects in the cell death machinery.

1.1.1 Apoptosis

Apoptosis refers to a special type of programmed cell death (PCD) characterized by unique features such as rounding-up of the cell, cell shrinkage, chromatin condensation, membrane blebbing, fragmentation of the nucleus and the break down of DNA into oligonucleosomes. The disassembled cell is packed into membrane enclosed apoptotic bodies which express special markers like the phosphotidyl serine (PS) groups to be recognised and phagocytosed by the neighbouring cells or the macrophages (Savill and Fadok, 2000).

1.1.1.1 Cellular and molecular basis of apoptosis

Apoptosis is a genetically and evolutionarily conserved and tightly controlled process. Apoptosis is induced by specific interaction of cell surface receptors with their ligands or by intracellular signalling pathways with extra- or intracellular inducers of apoptosis followed by serial activation of proteolytic caspases. The caspase activation

leads to cleavage of various cytoplasmic proteins, DNA repair enzymes followed by prevention of DNA repair, DNA fragmentation, and disruption of the cellular structure. The process of apoptosis can proceed through two major pathways, namely, intrinsic and extrinsic pathways to activate terminal caspase-3, -6 and -7. These pathways are not essentially exclusive as there are several caspase independent apoptosis-like PCDs recognised (Jaattela and Tschopp, 2003; Lockshin and Zakeri, 2002).

1.1.1.2 Caspases

In mammalian cells, apoptosis is executed by activation of multiple caspases (cysteinyl aspartate-specific proteases), which belong to a family of cysteine proteases. They recognise tetra-peptide motifs and specifically cleave after the aspartate residues in their plethora of substrates (Dixit, 1996; Earnshaw, et al., 1999) and this cleavage irreversibly modify their target protein function (Thornberry and Lazebnik, 1998). Currently 13 mammalian caspases have been identified and based on their function and length of the pro-domains they are classified in to three groups: inflammatory caspases, apoptotic initiator caspases and apoptotic effector caspases (Philchenkov, 2004).

Caspases are expressed as inactive zymogens (procaspases) that become activated by dimerization and/or autocatalytic cleavage. This activation is achieved by scaffolding proteins that either concentrate the zymogens sufficiently for autocatalysis ('induced proximity' model (Salvesen and Dixit, 1999)) or allosterically activate the zymogen without processing it (Rodriguez and Lazebnik, 1999). Inflammatory caspases include Caspase-1, -4, and -5, which are predominantly involved in the maturation of proinflammatory cytokines. The initiator caspases (including caspase-2, -8, -9 and -10) function as sensors and transducers of various apoptotic stimuli. They contain either a death effector domain (DED) or a caspase activation and recruitment domain (CARD), which are important for interactions with upstream adaptor molecules (Adams and Cory, 2002). Oligomerization of the initiator caspases through binding of adaptor molecules results in their activation. The activated initiator caspases subsequently cleave and activate the effector caspases (including caspase-3, -6 and -7), which in turn cleave multiple cellular substrates leading to the execution of apoptosis (Boatright, et al., 2003; Fischer, et al., 2003). In some cases, effector caspases can also function as initiator caspases; this activity helps to amplify a

suicide signal in the cell whose death pathways have been only weakly initiated. Furthermore, the activation of effector caspases can also be achieved by non-caspase proteases, including cathepsins, calpains, and granzymes (Johnson, 2000).

1.1.1.3 Apoptosis signalling pathways

Depending on the type of stimuli, apoptosis can be induced through two distinct pathways namely the "extrinsic" or receptor-mediated and the "intrinsic" or mitochondria-mediated pathways (Roy and Nicholson, 2000).

1.1.1.3.1 Extrinsic apoptosis

Death receptors situated on the plasma membrane are responsible for the induction of the extrinsic apoptotic-signalling pathway. Activation of the receptors by ligand binding induces a signalling cascade that culminates in degradation of the cell. There are eight death receptors characterized to date all of which belong to the tumor necrosis factor (TNF) receptor super family. These death receptors include TNF-R1 (DR1/CD120a/p55/p60), Fas (APO-1/CD95), death receptor (DR)3 (APO-3/LARD/TRAMP), TNF related apoptosis inducing ligand (TRAIL)-R1 (DR4/APO-2), TRAIL-R2 (DR5/KILLER/TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor (NGFR) (Bhardwaj and Aggarwal, 2003; Lavrik, et al., 2005). Members of this family contain one to five cysteine rich repeats in their extra cellular domain, and a death domain in their cytoplasmic tail (Krammer, 2000; Nagata, 1996; Nagata, 1996; Peter and Krammer, 2003).

1.1.1.3.1.1 Fas/CD95 signaling pathway

CD95 or Fas is one of the well-characterized members of the TNF receptor super family (Ashkenazi and Dixit, 1999; Lavrik, et al., 2005). Upon binding to the Fas (APO1) Ligand, the receptor trimerizes and recruits the adaptor protein FADD (Fas-Associated Death Domain) through their respective death domains (DD). Besides the DD, FADD also contains a DED (Death Effector Domain) that can bind to the DD found in caspase-8. Thus, binding of FADD to the Fas receptor will recruit and induce dimerization of caspase-8 resulting in DISC formation (Death-Inducing Signaling Complex), further leading to caspase-8 activation (Boatright, et al., 2003; Curtin and Cotter, 2003; Donepudi, et al., 2003). The proteolytically

active caspase-8 in turn activates down-stream effector caspases (caspase-3, -6 and -7), which subsequently execute the apoptotic program (Fischer, et al., 2003) (Figure 1).

Procaspase-10 can also bind to the DISC and become activated similar to caspase-8 activation upon Fas ligation. However, the importance of caspase-10 for apoptosis induction in the absence of caspase-8 is still controversial and needs to be elucidated (Sprick, et al., 2002).

1.1.1.3.1.2 TRAIL- and TNF-receptor signalling

TRAIL induced signalling via TRAIL-R1 and TRAIL-R2 is very similar to Fas signalling. However, TRAIL is also involved in non-apoptotic signalling, which proceeds via NF-kappaB, PKB/Akt and the MAPK signalling pathways (Falschlehner, et al., 2007). Whereas, the TNF-receptor signalling is more complex. The pleiotropic actions of TNF range from proliferative responses such as cell growth and differentiation, to inflammatory effects and the mediation of immune responses, to destructive cellular outcomes such as apoptotic and necrotic cell death mechanisms (MacEwan, 2002). Apoptosis induced by TNF-receptor 1 (TNF-R1) involves a two-step activation cascade. First, the membrane bound TNF- R1, the adaptor TRADD, TRAF2 and the kinase RIP1 (complex I) activates NF-kappa B and/or JNK. In a second step, TRADD and RIP1 associate with FADD and caspase-8, forming a cytoplasmic complex (complex II). Activated NF-kappa B mediates transcription of a caspase-8 inhibitory protein, cFLIP, which interferes with the apoptosis signalling pathway from TNF-R1 and assures survival of the cell. Thus, TNF-R1 signalling harbours a checkpoint were a decision between survival and death can be made (Micheau and Tschopp, 2003). cFLIP is also active in the CD95 / Fas and the TRAIL pathways.

1.1.1.3.2 Intrinsic apoptosis

The intrinsic apoptotic pathway is initiated within the cell and involves permeabilization of the mitochondria (Figure 1). This pathway is triggered by various stress signals, such as DNA damage mediated by ionising irradiation or chemotherapeutic agents. Stress signals elicit various signalling cascade reaching on to mitochondria to induce mitochondrial outer membrane permeabilization (MOMP). The local regulation and execution of MOMP involves proteins from the Bcl-2 family (Bax, Bak, and, in some cases, Bim, tBid and Puma) (Green and Kroemer, 2004). MOMP, in turn, causes apoptosis via the

release of apoptogenic molecules like cytochrome c from the mitochondria into the cytoplasm. Cytochrome c, which normally has an important function in the respiratory chain (Liu, et al., 1996), assembles together with the adaptor molecule, APAF-1, ATP and caspase-9 into a complex called the apoptosome. This leads to aggregation and activation of caspase-9, which subsequently cleaves and activates downstream effector caspases (caspase-3, -6 and -7), which then execute cell death. Thus, downstream signalling from initiator caspases is the same for the intrinsic and extrinsic pathways. Several other pro-apoptotic proteins, such as AIF, EndoG, Smac/Diablo and HtrA2/Omi are also released from the mitochondria. Apart from mitochondria, accumulating evidence strongly suggests a significant involvement for other organelles like the lysosomes, endoplasmic reticulum and Golgi during apoptosis as well (Ferri and Kroemer, 2001).

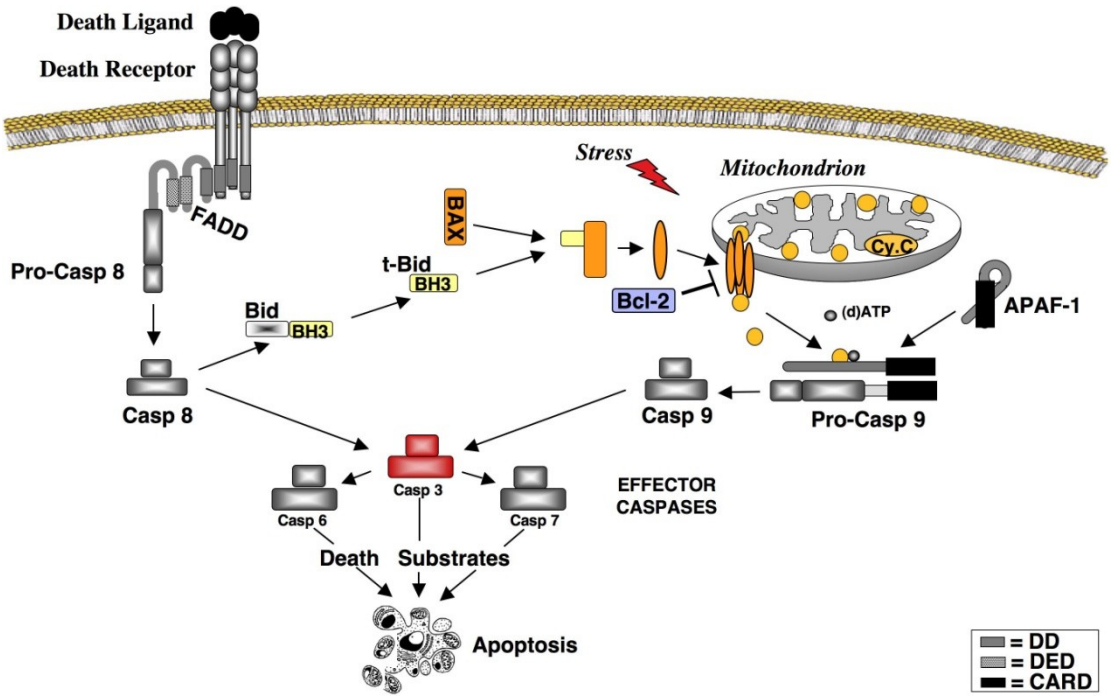

Figure 1: Extrinsic and Intrinsic apoptosis pathway and crosstalk. Shown in the left side is the schematic reperesentation of the death receptors-mediated extrinsic signalling pathway of Fas. Shown in the right side is the Schematic reperesentation of the mitochondrial-mediated intrinsic signalling pathway. The extrinsic and intrinsic pathways can connect at different levels. I) Active caspase-8 can cleave Bid, which in its truncated form (tBid) activates the intrinsic pathway. Activation of the intrinsic pathway by generation of tBid can serve as an amplification loop for extrinsic signalling in cells with low levels of active caspase-8. II) Activation of the intrinsic pathway can result in the activation of caspase-8, which enhances the intrinsic apoptotic response.

1.1.1.3.3 Cross-talk between the extrinsic and intrinsic pathways

Two models of Fas signalling have been described by which cells can be classified as type I or type II, depending on the engagement of the intrinsic pathway (Barnhart, et al., 2003). Type I cells have high levels of Fas-induced DISC formation and consequently high levels of activated caspase-8, which is sufficient for sustained caspase-3 activation. On the contrary, low amount of DISC formation and low levels of caspase-8 activation characterize type II cells. Therefore, amplification of the signal via activation of the intrinsic pathway is needed. Low amounts of caspase-8 are sufficient to cleave Bid into an active truncated form (tBid). Bid is a pro-apoptotic member of the Bcl-2 family that, upon cleavage, translocates to the mitochondrial membrane and stimulates processes to enable cytochrome c release and subsequent events (Gross, et al., 1999). Caspase-8 activation can be induced during intrinsic apoptosis signalling (Scaffidi, et al., 1998) and it has been shown to accelerate the intrinsic death response (Tang, et al., 2000). In some cases, activation of caspase-8 occurs independently of the CD95/Fas death-inducing signalling complex and down-stream of the main executioner of the apoptosis signalling complexes, caspase-3, where activated caspase-3 cleaves caspase-8 in a feed-back loop manner (Wieder, et al., 2001). Furthermore, activated caspase-6 has been reported to be an activator of caspase-8 (Cowling and Downward, 2002). In summary, it is evident that crosstalks exist between the extrinsic and intrinsic apoptosis signalling pathways and they function as amplification loops for each cascade (Figure 1).

1.1.1.4 Regulation of apoptosis signalling

When considering the physiological importance of apoptotic responses, it is clear that tight regulations of the signalling cascades are needed. Several regulators of death receptor mediated extrinsic and mitochondria-mediated intrinsic signalling have been identified and they are briefly described below because of their relevance for apoptosis resistance.

1.1.1.4.1 Regulation of death receptor mediated extrinsic signalling

One of the prime regulators of the death receptor signalling belong to a family of endogenous inhibitors called the cellular FLICE-like inhibitory proteins (cFLIPs). In 1997,

investigators identified the viral protein, vFLIP, while screening for DED-containing proteins that could interact with caspases (Thome, et al., 1997). The mammalian homologue is cFLIP, which is also refered to as cellular FLIP, CASH, FLAME, Casper, CLARP; FLAME, I-FLICE, MRIT and usurpin by several other groups (Budd, et al., 2006).

There are several splice variants of cFLIP reported but only three of them have been identified to be expressed as proteins. cFLIP-Long (cFLIP-$_L$) is similar to caspase-8, as it contains two N-terminal DEDs and a caspase-like domain. However several important amino acids are different in the caspase-like domain of cFLIP-$_L$, which abolishes the enzymatic activity. The two other cFLIP splice variants, cFLIP-Short (cFLIP-$_S$) and cFLIP-Raji (cFLIP-$_R$) are truncated versions, which contain only the two DEDs. All three cFLIP isoforms act as competitive inhibitors of caspase-8 for FADD binding thereby inhibiting Fas induced apoptosis (Golks, et al., 2005; Irmler, et al., 1997; Scaffidi, et al., 1999). c-FLIP-$_S$ completely inhibits cleavage of procaspase-8 (Krueger, et al., 2001; Peter, 2004) whereas, cFLIP-$_L$ function is more complex. Although cFLIP-$_L$ can compete with caspase-8 for recruitment to the DISC and can therefore inhibit caspase-8 activation, it also forms heterodimers with caspase-8 at the DISC, which leads to week activation and initial processing of caspase-8 that result in the release of the p10 fragment of caspase-8. Association of cFLIP-$_L$ with caspase-8 also mediates processing of cFLIP-$_L$, which generates 43kDa and 12kDa products from the 55kDa full-length protein (Krueger, et al., 2001). Interestingly, the p43 cFLIP fragment acts as a more potent activator of NFkappaB than full-length cFLIP-$_L$ (Kataoka and Tschopp, 2004) besides inhibition of Fas induced apoptosis through interfering with caspase-8 cleavage. However, the role of cFLIP-$_L$ in NFkappaB activation is complex and contradicting studies exist where cFLIP-$_L$ has also been shown to inhibit NFkappaB activation (Kreuz, et al., 2004; Legembre, et al., 2004). Nevertheless cFLIP exerts discrete regulatory functions involving inhibition of caspase-8 induced apoptosis, and also in other signalling cascades such as survival pathways from the death receptors.

1.1.1.4.2 Regulation of mitochondrially-mediated intrinsic apoptosis signalling by Bcl-2 family members

Bcl-2-family proteins play an important role in regulating the intrinsic apoptotic pathway. The Bcl-2 (B-cell lymphoma-2) gene was the first anti-apoptotic gene to be

discovered as the defining oncogene in B-cell follicular lymphomas and is located at the chromosomal breakpoint of the t(14;18) (q32;q21) chromosomal translocation (Danial, 2007; Tsujimoto, et al., 1984). To date the family consists of 20 members, which can be divided into three subfamilies based on the presence of conserved Bcl-2 homology (BH1-4) domains (Youle and Strasser, 2008). One subfamily is constituted of anti-apoptotic proteins, such as Bcl-2, Bcl-x_L (Bcl-2 related X gene, long isoform), Bcl-w, Mcl-1 (Myeloid cell leukemia 1), Bfl-1 and Bcl-B which contain BH domains 1–4 and are generally integrated within the outer mitochondrial membrane, but there are instances of cytosolic, endoplasmic and nuclear membrane localizations (Kaufmann, et al., 2003). The other two subfamilies include the pro-apoptotic proteins. Bak (Bcl-2 antagonist killer 1), Bax (Bcl-2 associated x protein) and Bok (Bcl2-related ovarian killer) are three important members of the Bax-like apoptotic subfamily. These proteins contain BH1-3 domains and they are responsible for mitochondrial membrane permeblization and cytochrome c release. The third subfamily is the more diverse pro-apoptotic BH3 only family and it includes Bad (Bcl-2 antagonist of cell death), Bid (Bcl-2 interacting domain death agonist), Nbk/Bik (Natural born killer/Bcl-2 interacting killer), Bim (Bcl-2 interacting mediator of cell death), Bmf (Bcl-2 modifying factor), Bnip3 (Bcl-2/adenovirus E1B 19-KD protein-interacting protein 3), Hrk (Harakiri), Noxa (Latin for damage) and Puma (p53-upregulated modulator of apoptosis), which function in distinct cellular stress pathways, by protein–protein interactions with other Bcl-2 family members (i.e. anti-apoptotic Bcl-2 proteins and/or the effector molecules Bax, Bak or Bok), and signal that a cellular stress has occurred (See Figure 2 for overview) (Daniel, 2000).

The levels and activity of pro-survival proteins Bcl-x_L, Bfl-1 and Mcl-1 are regulated by diverse mechanisms, including transcriptional control and protein modification and turnover. For example, the levels and activity of pro-survival proteins are closely coupled to the supply of cytokines, which affects both their production and stability. Bcl-2 levels may also be controlled, in part, by micro-RNAs (Cimmino, et al., 2005; Xia, et al., 2008) and its activity is affected in complex ways by phosphorylation (Deng, et al., 2004). DNA damage induces deamination of Bcl-x_L on two asparagine residues in its flexible loop region (Deverman et al., 2002), but the claim that this blocks Bcl-x_L pro-survival activity may well be erroneous (Deverman et al., 2003). The Mcl-1 protein is rapidly lost by proteasomal degradation early in response to several cytotoxic signals (Cuconati, et al., 2003). Proteins

implicated in the regulation of its degradation include Noxa (Willis, et al., 2005), a BH3-containing E3 ubiquitin ligase called Mule (Zhong, et al., 2005) and β-TrCP (Ding, et al., 2007).

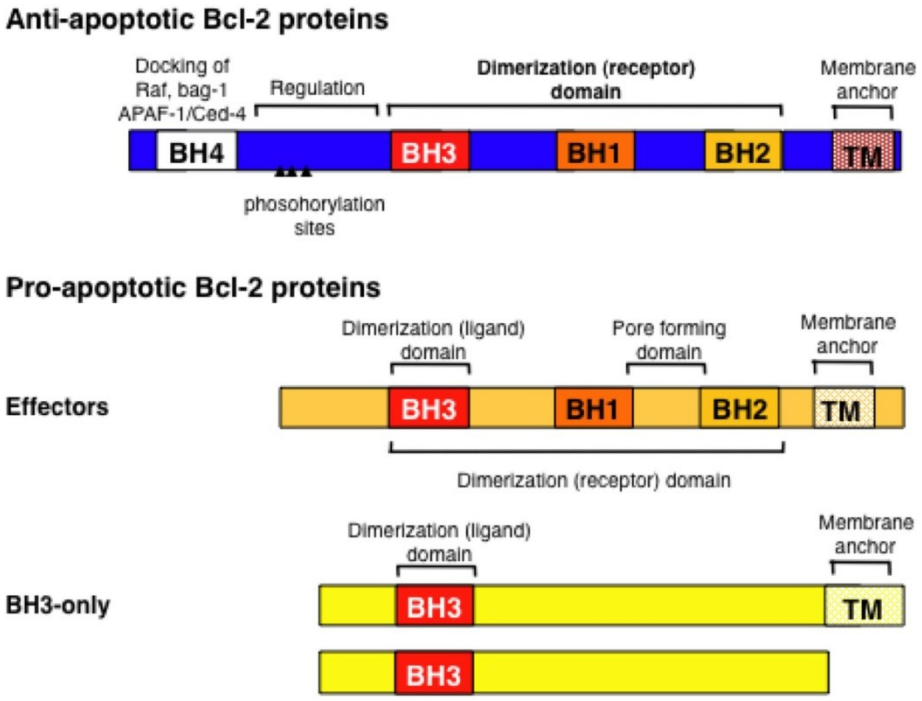

Figure 2: The Bcl-2 family members and their function at the mitochondrial membrane. The Bcl-2 family proteins can be divided into three subgroups depending on how many BH-domains they contain. All four BH-domains are found in the anti-apoptotic Bcl-2 like subfamily, whereas the Bax-like subfamily of the pro-apoptotic proteins only contains three BH-domains. The third subfamily consists of the BH3 only proteins, which along with the Bax-like subfamily function as apoptosis inducers. Several proteins in all the subgroups also contain a transmembrane domain (TM), which affects the protein function by anchoring them to intracellular membranes like the mitochondria, nuclear envelope and endoplasmatic reticulum**.**

Both pro-apoptotic groups of the Bcl-2 proteins are required for stress-induced apoptosis. The BH3-only proteins function as damage sensors upstream of Bak and Bax, as Bim, Bad, Noxa and Nbk failed to induce apoptosis in Bax and Bak double deficient cells (Cheng, et al., 2001; Gillissen, et al., 2003; Gillissen, et al., 2007; Zong, et al., 2001). Some BH3-only proteins are activated by transcriptional induction (Puma, Noxa) or post-translational modifications, like dephosphorylation (Bad) (del Peso, et al., 1997; Zha, et al., 1996) and cleavage (tBid) (Li, et al., 1998; Luo, et al., 1998). Whereas Bim and Bmf are initially associated with the microtubules and the actin cytoskeleton respectively via interaction with a Dynein Light Chain (DLC) their release can induce apoptosis (Puthalakath et al., 1999; Puthalakath et al., 2001). Bim is identified to be one of the principle regulators

of haematopoietic homeostasis: In its absence, leukocyte numbers rise and plasma cell accumulation leads to the onset of an autoimmune syndrome equivalent to that elicited by the overexpression of Bcl-2 (Bouillet, et al., 2002; Bouillet and Strasser, 2002). The level of Bim can be regulated by phosphorylation by Erk, which triggers its degradation by the proteasome (Akiyama, et al., 2003; Ley, et al., 2003; Luciano, et al., 2003), whereas its phosphorylation by c-Jun N-terminal kinase may potentiate its pro-apoptotic activity (Putcha, et al., 2003).

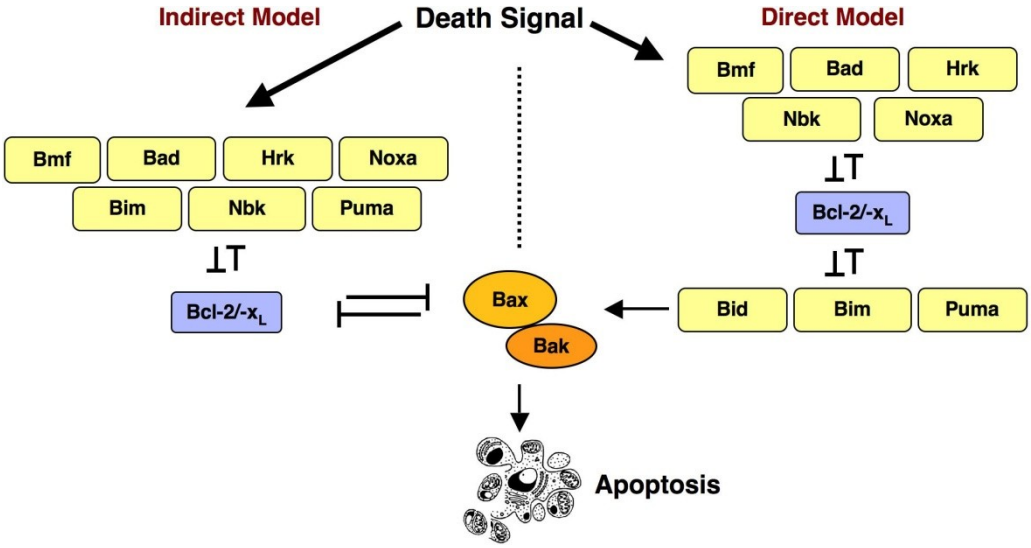

Figure 3: Regulation of intrinsic apoptosis pathway by BH-3 only proteins. Various stress stimuli result in the activation of the BH3-only proteins, which function as apoptotic sensors in the cytoplasm. Subsequently, in indirect model BH3 proteins, Bim, Bid and Puma bind to Bcl-2 pro-survival proteins leading to disengagement and activation of Bax/Bak for apoptosis induction. In the direct model the BH3 only proteins activate the Bax-like protein directly by changing their conformation whereas the indirectly model is based on liberating the inhibitory binding to anti-apoptotic Bcl-2 proteins. Activation of the Bax-like proteins leads to their oligomerization at the mitochondrial membrane, which results in the release of pro-apoptotic proteins into the cytoplasm.

Active BH3-only proteins mediate conformational change in Bax and Bak, which induces protein oligomerization at the outer mitochondrial membrane, thus leading to MOMP and cytochrome c release. There are two models proposed for the activation of Bak and Bax by the BH3-only proteins (Figure 3) (Youle and Strasser, 2008). In the first model, a direct association between Bak/Bax and certain BH3-only proteins, i.e. Bim, tBid and Puma has been described (Adams and Cory, 2007; Certo, et al., 2006; Kuwana, et al., 2005; Letai,

et al., 2002; Oh, et al., 2006; Walensky, et al., 2006). In this model, the rest of the BH3-only proteins are suggested to bind anti-apoptotic proteins and abolish their inhibitory interaction with Bim, tBid and Puma. In the indirect activation model, the BH3-only proteins are believed to induce apoptosis merely by interaction with anti-apoptotic proteins, thus preventing them from inhibiting Bax and Bak activation (Chen, et al., 2005; Willis, et al., 2005; Willis, et al., 2007). The functional relevance of these two activation models is currently debated and it is likely that they are of different importance in different cellular systems (Adams and Cory, 2007).

1.1.1.4.3 Regulation of apoptosis by IAPs

Apoptosis including both extrinsic and intrinsic signalling pathways is under the control of the inhibitors of apoptosis, IAPs. These proteins were initially identified in baculovirus (Crook, et al., 1993) and they are characterized based on the presence of one to three baculoviral IAP repeats (BIRs). There are at least 8 different human IAPs identified (Survivin, ILP2, ML-IAP, XIAP, c-IAP1, c-IAP2, NAIP and BRUCE). Structural and functional studies have shown that the IAPs can bind directly to different caspases, mainly caspase-9, -3 and -7, and inhibit their activity (Richter and Duckett, 2000; Salvesen and Duckett, 2002). The inhibitory effect is mediated by binding of the BIR domains to the caspases, which sequesters the caspases away from their targets and/or induce their degradation (Srinivasula and Ashwell, 2008).

1.1.1.4.4 Regulation of apoptosis by p53

The p53 transcription factor is often called the guardian of the genome and it has a pivotal role in prevention of tumor formation. Mutations, deletions or dysregulation of p53 are some of the most common alterations detected in tumors. The expression levels of p53 is generally maintained low due to its binding to mdm2, which targets p53 for proteasomal degradation (Momand, et al., 2000). DNA damage and cellular stress induces p53 stabilisation and this together with specific modifications of p53, such as phosphorylations, acetylations and glycosylations, leads to its activation (Vogelstein, et al., 2000). p53 controls a large number of genes transcriptionally that influence cell cycle arrest, DNA repair, apoptosis, senescence and autophagy. It has been established as one

of the most important DNA damage cell cycle checkpoint proteins. For instance, it has a major regulatory role in response to radiation (Pawlik and Keyomarsi, 2004). The apoptotic function of p53 is mainly executed through activation of pro-apoptotic proteins, such as Bax, Noxa, Puma, Bid, Fas, APAF-1 and TRAIL-R2. Additionally, many studies have shown that p53 mediates transcriptional repression of anti-apoptotic genes like Bcl-2 and survivin (Haldar, et al., 1994; Hoffman, et al., 2002). Notably, p53 has also been reported to impede the anti-apoptotic function of Bcl-2 and Bcl-x_L as well as to activate the pore- forming function of Bax and Bak by a direct binding (Leu, et al., 2004; Norbury and Zhivotovsky, 2004) Nevertheless, cytosolic or mitochondrial p53 is, per se, not sufficient to activate the intrinsic apoptosis pathway (Essmann, et al., 2005).

1.1.2 Non-apoptotic cell death

1.1.2.1 Necrosis

Necrotic cell death is an unsheduled accidental cell lysis, which has been often regarded opposite to orderly execution of apoptosis. It is characterized by swelling of organelles and cytoplasm that eventually leads to disruption of the plasma membrane and is considered to be an energy-independent and un-regulated form of cell death (Leist and Nicotera, 1997; Nicotera and Leist, 1997). Normally, an inflammatory response is generated as the content of the cells leaks out to the surrounding tissue. Necrosis is often induced by acute and severe injuries, like in pathological conditions and ischemia (Galluzzi, et al., 2007). Recently, evidence has emerged showing that necrosis, under certain conditions, can be programmed which is cited as necroptosis. For instance, Arsenic trioxide induces regulated, death receptor- and caspase-independent cell death through a Bcl-2-controlled pathway (Degterev and Yuan, 2008; Scholz, et al., 2005; Scholz, et al., 2005). In case of impaired normal apoptosis signalling, induction of necrosis can be an important alternative means of cell death. Though the signalling mechanisms leading to necrosis are not fully understood, there are some important findings made. For instance, in the absence of caspase-8, Fas induces necrosis through an effector molecule RIP-1 kinase, (Holler, et al., 2000) and calcium as well as reactive oxygen species are shown to play an important role in the execution of necrosis (Festjens, et al., 2006).

1.1.2.2 Autophagy

Autophagy is an evolutionary conserved "self-eating" process that eliminates damaged or dysfunctional cell components. It is essential for cellular homeostasis, survival, development and differentiation. Normally, autophagy is a slow process, but it can serve as an adaptive response against several pathologies like infection, neurodegeneration and cancer. Several different forms of autophagy have been described, namely macro-, micro- and chaperone-mediated autophagy (CMA). Macro- and micro-autophagies are capable of degrading large structures, whereas CMA is only concerned about soluble proteins. Macroautophagy occurs at a basal level in response to environmental conditions like nutrients deprivation or microbial pathogens, during which double-membrane-containing vesicles are formed that are called phagosomes. These phagosomes enclose organelles and proteins, which are subsequently degraded when the vesicles fuse with lysosomes. In contrast, the micro-autophagy and CMA involves direct incorporation of unfolded substrate proteins into the lysosomes through the action of a cytosolic and lysosomal chaperone hsc70 along with the integral membrane receptor LAMP-2A (lysosome-associated membrane protein type) (Mizushima, et al., 2008). Autophagy is under the control of a large family of autophagy-related genes (ATG). During stress conditions like nutrient and growth factor deprivation, autophagy can sustain cell survival by degrading disposable cell components (Levine and Kroemer, 2008). In case of uncontrolled up-regulation of autophagy the outcome can be cell death. In case of uncontrolled up-regulation of autophagy the outcome can be cell death. For instance, over expression of ATGs, e.g. beclin-1, leads to cell death in mammalian cells (Pattingre, et al., 2005). Autophagy might therefore keep damaged cells alive which can lead to potential danger supporting pathologies like tumor formation (Levine and Kroemer, 2008).

1.1.2.3 Mitotic catastrophe

Mitotic catastrophe (MC) refers to cell death that occurs during mitosis or caused by mitotic failure (Figure 4). Other investigators consider mitotic catastrophe not as a mode of cell death but as a special case of apoptosis which is based on the observation that MC shares several biochemical hallmarks with apoptosis, namely mitochondrial membrane permeabilization and caspase activation (Bataller and Portugal, 2005; Castedo and Kroemer,

2004). However, it is still ambiguous whether death associated with MC occurs via caspase-dependent or caspase-independent mechanisms. In some studies, MC is related to a cell survival mechanism of tumors (Mansilla, et al., 2006); (Erenpreisa, et al., 2005), while others interpret that MC is associated with senescence (Eom, et al., 2005; Shay and Roninson, 2004). Despite all these definitions, there is no generally accepted characteristic of MC. Nevertheless, MC is often associated with the formation of giant cells with altered nuclear morphology, i.e. multiple nuclei or micronuclei (Galluzzi, et al., 2007) as a result of abnormal mitosis through the generation of lagging chromosomal material, anaphase bridging or multiple spindle poles. These cells may continue to divide and become polypoid and/or aneuploid, but eventually they die by delayed apoptosis or necrosis (Vakifahmetoglu, et al., 2008). Mitotic catastrophe usually results from defects mediated by impaired cell cycle checkpoints (Castedo, et al., 2004). Therefore, mitotic catastrophe is particularly prevalent in cells with compromised p53 function, as p53 is a major regulator of both G1 and G2 checkpoints (Pawlik and Keyomarsi, 2004). Suppression of the spindle checkpoint, p53 function and the apoptotic program may thus lead to mitotic slippage and asymmetric cell division, resulting in the generation of tetraploid (4N) cells which can further result in aneuploid offspring, if the polyploidy checkpoint is inactivated (Bhonde, et al., 2006; Rieder and Maiato, 2004). In addition, it can be caused by anomalous duplication of centrosomes, as these are crucial for the number of spindle poles formed during mitosis and for accurate chromosome segregation into daughter cells (Loffler, et al., 2006). In general, resistance to cell death by mitotic catastrophe mainly depends on the molecular profile of the cell and the extent of DNA damage that will ultimately define if the cell will enter G1 as a viable entity. It is tempting to speculate that these mechanisms could be important for cancer development.

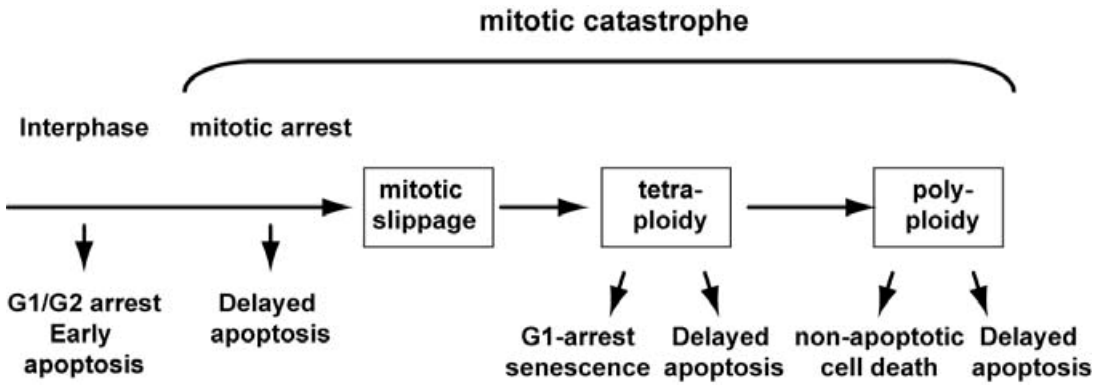

Figure 4: Mitotic catastrophe is a cell death induced in mitosis or as a consequence of mitotic abnormalitites. It can be detected at multiple stages during the accumulation of DNA alterations depending on the genetic backround of the cell and the intensity of the DNA damaging stimuli applied. (Modified from Eriksson et.al. 2008).

1.2 Apoptosis resistance in malignant disease

Acquired resistance towards apoptosis can be observed in most types of cancers, which supports tumor development in a profound way. There are numerous molecular alterations described that promotes both tumor development and progression. It is well established that mutations of important tumor suppressor genes and oncogenes like p53, c-Myc, Ras, Wnt and Erk act as a major driving force for cellular transformation. These mutations can occur through exchange of single nucleotides, reading frame shifts or rearrangements of larger DNA stretches, such as deletions, inversions and translocations (Sjoblom, 2008). Epigenetic alterations, like DNA methylation and histone modifications, were also appreciated to have a great influence on tumor development (Vucic, et al., 2008). A significant number of proteins involved in the induction and regulation of cell death has been reported to be modified in various types of cancer which includes reduced function of pro-apoptotic molecules (for example: Bax, Fas, TRAIL-R1/2, Caspase-8, etc) and increased expression of anti-apoptotic proteins (for example: Bcl-2, Mcl-1, c-IAP2, Survivin, etc) (Daniel, et al., 1999; Igney and Krammer, 2002; Radetzki, et al., 2002; Raisova, et al., 2001; Sturm, et al., 1999; Sturm, et al., 2006; Zantl, et al., 2007).

1.2.1 Apoptosis resistance confers insensitivity to cancer therapy

In addition to the apoptosis resistance acquired during tumor formation and

progression, tumor cells also acquire resistance during treatment. The development of resistance to the drugs originally used to treat them is arduous, as it might contribute to cross-resistance against additional chemotherapeutics with different mode of action (Longley and Johnston, 2005). Recently, novel strategies have been taken to find specific targets in the apoptosis signalling pathways that, upon altered regulation, would promote apoptosis in tumor cells. Some promising therapeutic candidates include activating substances of molecules in the death signalling pathways (for instance: TNF-R1, TRAIL-R1, TRAIL-R2, etc) as well as drugs that interfere with the anti-apoptotic properties of survival proteins (for example: mTOR, PI3 kinase, Bcl-2, Bcl-x_L, survivin, IAPs, etc) (Ghobrial, et al., 2005; Ziegler and Kung, 2008).

1.3 The Cell Cycle

The cell cycle or cell division cycle is the series of events that take place in a cell leading to its division and duplication (replication) by mitosis unlike in cells without a nucleus (prokaryotes), where the cell cycle division occurs via a process termed binary fission. The cell-division cycle is a vital process by which a single-celled fertilized egg develops into a mature organism, as well as the process by which, hair, skin, blood cells and some internal organs are renewed.

1.3.1 The cell cycle phases

The cell cycle is divided into four different phases: G1, S, G2 and M phase (Fig. 5). The G1 and G2 phases are gaps during which the cell is preparing for DNA synthesis (S) and mitosis (M) respectively (Figure 5). Following mitosis, the cells may enter a quiescent G0 phase in the absence of stimuli triggering mitosis, a state that characterizes most of the cells in normal adult tissues. In the presence of a sustained mitogenic stimulus, the cells progress to a restriction point (R), beyond which they are committed to enter S phase and growth factors present in the environment are no longer required for progression in S/G2 phases (Sherr, 1994). In vertebrates and diploid yeasts, the cells in G1 have a diploid number of chromosomes (2N), one inherited from each parent, in S when DNA is replicated it is between 2N and 4N and in G2/M the cells are tetraploids with a 4N chromosome number.

Mitosis is further sub-divided into four phases based on morphological criteria, the

prophase characterized by chromosome condensation, the metaphase where chromosomes align in the cell center, the anaphase when sister chromatids separate and move to the opposite poles of the mitotic spindle and the last stage, the telophase, where the segregated chromosomes decondense followed by physical division of the cytoplasm that yields two daughter cells called cytokinesis. During this, the nuclear envelope breaks down into vesicles in prophase and reforms again in telophase. Besides the Golgi complex and endoplasmic reticulum vesiculate during mitosis and reform in the daughter cells when cell division is completed.

1.3.2 Regulation of the cell cycle

Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. Progression through the cell cycle is regulated by the synthesis, assembly and activation of key cell cycle regulatory complexes comprised of cyclins and cyclin dependent kinases (Cdks), followed by their subsequent inactivation, disassociation and degradation in a specific order (Morgan, 1995). This process involves multiple mechanisms controlling at transcriptional, translational and post-translational levels via the ubiquitin-mediated proteolysis and regulation of the subcellular localization of proteins.

1.3.3 Cyclin dependent kinases (Cdks) and cyclins

In mammalians, the Cdks comprise a family of eleven serine-threonine protein kinases (Cdk 1-11) and four of them were shown to catalyze different cell cycle transitions (Pfleger and Kirschner, 2000). Cdk4 and Cdk6 are active during G1, Cdk2 in the G1/S transition and Cdk1 in late G2 and mitosis. Binding of cyclin to Cdk is an absolute requirement for their activation (Sherr, 1994). Activation of Cdk's requires phosphorylation in a conserved threonine residue located in their activation loop that causes a conformational change and is essential for the kinase activity. This phosphorylation event is catalysed regulatorily by various kinases like Cdk activating kinase (CAK) (Sherr, 2000). Phosphorylation at conserved inhibitory sites (threonine 14 and tyrosine 15) on Cdk1/Cdc2 inhibits their activity and is catalysed by the Wee-1 and Myt-1 kinases. These inhibitory phosphates are removed by another family of regulators, the cell division cycle (Cdc)

phosphatases including Cdc25A, Cdc25B and Cdc25C, leading to their full activation (Morgan, 1995). Apart from site specific phosphorylation, the Cdk activity is also negatively regulated by binding of Cdk inhibitors (CKI) belonging to two distinct families, the kinase inhibitory proteins (KIP) and the inhibitor of Cdk4 (INK4) (Sherr, 2000). The KIP family includes the p21, p27 and p57 proteins that are able to inhibit all cyclin–CDK complexes and are not specific for a particular phase (Slingerland and Pagano, 2000). While the members of the INK4 family including p15, p16, p18 and p19, specifically act in G1 and inactivate Cdk4 and Cdk6 by destabilizing their association with the D-type cyclins (Sherr and Roberts, 1999).

The family of mammalian cyclins include cyclins A to H and all share a conserved sequence of about 100 amino acids referred to as the cyclin box. Different cyclins bind to and activate different Cdks, at specific phases of cell cycle and the activated cyclin-Cdk complexes in turn phosphorylate various target proteins in order to mediate transition and progression through the different cell cycle phases. The levels of cyclin changes significantly during cell cycle unlike Cdks, which remain constant thoughout cell cycle. Entry and progression through G1 is regulated by the three D-type cyclins (cyclin D1, D2, and D3) that bind to Cdk4 and Cdk6 whereas entry into S phase is regulated by the cyclin E/Cdk2 complexes (Schafer, 1998). Cyclin A/Cdk2 complex is involved in S phase progression whereas in late G2 cyclin A binds to Cdk1. Entry from G2 to M and progression through early M phase, is controlled by the cyclin A/Cdk1 and cyclin B/Cdk1 complex (Figure 5).

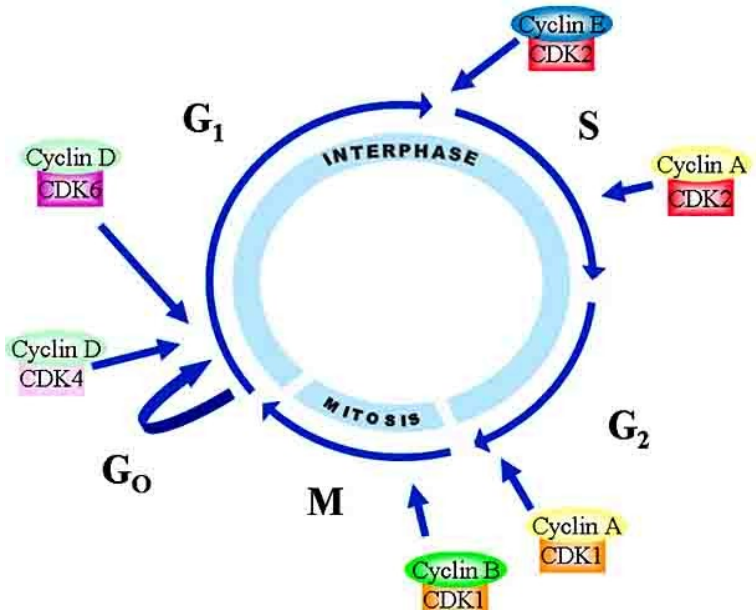

Figure 5 The cell cycle and its regulation by CDKs. In normally cycling cells, the cell cycle is composed of four main stages (G1, S, G2, and M) under the direction of cyclin dependent kinases (CDKs).

Key events of the cell division are also controlled by the ubiquitin-proteasome pathway either using SCF (Skp1/Cullin/F-box) E3-ligase principally at the G1/S and G2/M transitions (DeSalle and Pagano, 2001) or the anaphase-promoting complex (APC) at M phase for exit and entry in G1 phase (Hagting, et al., 2002; Salah and Nasmyth, 2000).

1.3.4 Cell cycle checkpoints and their role in tumor formation

The cell cycle checkpoints are surveillance mechanisms that ensure fidility of the cell cycle. When errors are detected they arrest the cell cycle to allow sufficient time to correct these errors. Defects in many molecules regulating the cell cycle have been implicated in cancer formation and progression (Kastan and Bartek, 2004). Among these the most crucial are p53, the retinoblastoma protein (pRb) and its related proteins, p107 and pRb2/p130, and cdk inhibitors (p15, p16, p18, p19, p21, p27), all of which act to keep the cell cycle from progressing until all repairs to damaged DNA have been completed (Macaluso, et al., 2005). There are also other proteins, which play important role in cell cycle progression specifically during mitosis such as Bub1, BubR1, PLK's (Polo like kinases) and the Aurora kinases. Alterations in cell cycle components due to mutations resulting in loss or gain of function may cause the cell to multiply uncontrollably, forming a tumor. The cells that are actively undergoing cell cycle are targeted in cancer therapy, as the DNA is relatively exposed during

cell division and hence susceptible to damage by drugs or ionising radiation. Notebly, PLK1 has also begun to attract increasing attention as it is overexpressed in a variety of human cancers, and its expression often correlates with poor disease prognosis (Eckerdt, et al., 2005).

1.3.4.1 Structural and functional aspects of PLK1

Polo, a serine/threonine kinase belongs to the Polo Like Kinase (PLK) family, which was first identified in *Drosophila melanogaster* (Sunkel and Glover, 1988). Subsequently other groups identified four mammalian PLK family members - PLK1, PLK2 (also known as SNK), PLK3 (also known as FNK or PRK) and PLK4 (also known as SAK) (Strebhardt and Ullrich, 2006). PLK1 is a well characterized member of the human PLK family which contains two domains: a highly conserved N-terminal catalytic (Kinase) domain of 252 amino-acids, and a C-terminal region with the unique PBD comprising 2 polo-boxes, each of which are 60–70 amino acids in length. Substrate specificity of PLK1 is modelled that PBD binding to target protein leads to a confirmational change of PLK1 liberating the kinase domain to phosphorylate the target protein (Lowery, et al., 2005). The activity and cellular concentrations of this kinase are crucial for the precise regulation of cell division. Along with the increase in PLK1 levels there is a concordant increase in kinase activity during G2 phase reaching to a maximum in mitosis at the metaphase-anaphase transition (Golsteyn, et al., 1995; Golsteyn, et al., 1994; Hardy and Pautz, 1996).

There are various functions associated with human PLK1: in centrosome maturation and spindle assembly (Lane and Nigg, 1996; Liu and Erikson, 2002; Sumara, et al., 2004; van Vugt, et al., 2004), and in facilitating proper bipolar spindle formation during prometaphase and metaphase (Figure 6). In the absence of PLK1, cells fail to form a bipolar spindle and arrest in a prometaphase-like state with an activated mitotic spindle checkpoint (Sumara, et al., 2004; van Vugt, et al., 2004). PLK1 has been implicated in chromatid arm separation through cohesin removal (Gimenez-Abian, et al., 2004; Hauf, et al., 2005; Sumara, et al., 2002). Additionally, it assists in mitotic exit and cytokinesis (Carmena, et al., 1998; Descombes and Nigg, 1998; Lindon and Pines, 2004; Seong, et al., 2002; Xie, et al., 2005). Notably, PLK1 has a significant role in responses and adaption to DNA damage (Smits, et al., 2000; Toczyski, et al., 1997; van Vugt, et al., 2004).

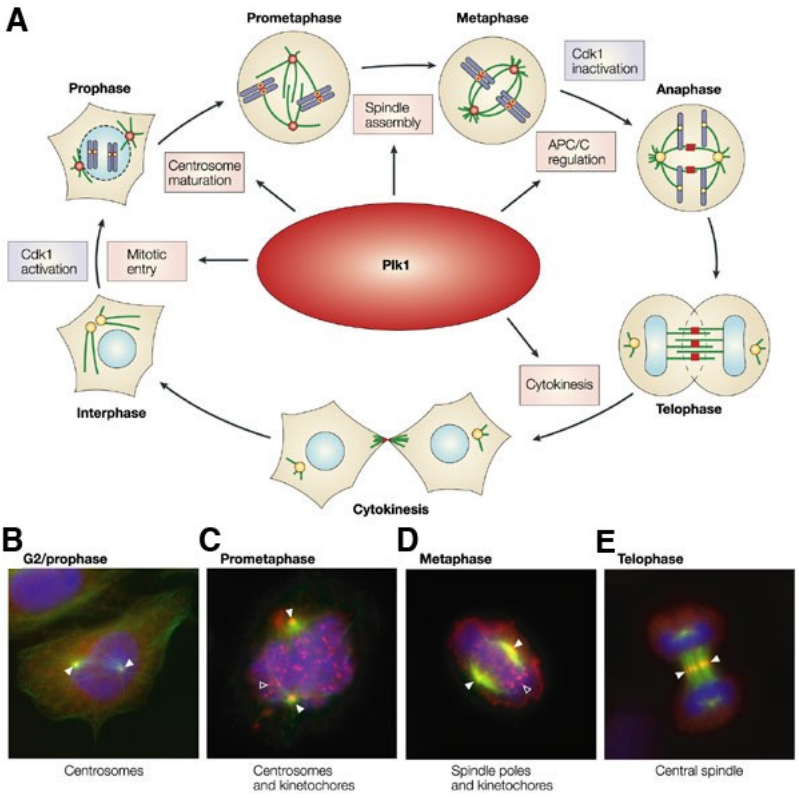

Figure 6: (a) Schematic view, emphasizing the function of polo-like kinase-1 (PLK1) at multiple stages of cell division. DNA/chromosomes are marked in blue, microtubules in green and both centrosomes and kinetochores in yellow. The red colour is used to indicate the association of PLK1 with different structures. (b–e) Immunofluorescence images, illustrating the various locations of PLK1 throughout the (HeLa) cell cycle. In G2, PLK1 localizes to centrosomes (b, filled triangles), then in prometaphase (c) and metaphase (d) to kinetochores (open triangles) and spindle poles (filled triangles), and finally, in anaphase–telophase, to the central spindle (e, filled triangles (Colocalization of PLK1 with tubulin results in yellow). PLK1 localization depends on a functional polo-box domain (PBD) and probably involves different docking proteins (Barr, et al., 2004).

On the other hand, studies in *X. laevis* oocytes described good evidence that PLKs bind and phosphorylate Cdc25, which might provide a mechanism for coordinating the regulation of Cdk1-cyclinB activity (Gonzalez, et al., 1998; Kumagai and Dunphy, 1996). Supporting this, Myt1 was identified as PLK substrate (Nakajima, et al., 2003). PLK1 also phosphorylates cyclin B1 on Ser133 targeting it to the nucleus during prophase (Toyoshima-Morimoto, et al., 2001) (Figure 7). This indicates that PLKs might have an essential role in the control of G2/M-progression (Barr, et al., 2004). DNA damage or failure to complete DNA

replication results in the activation of DNA-damage checkpoints, which inhibits Cdc25 and/or activate Wee1/Myt1, moreover, suppress PLK1 activity. The mechanisms that mediate the downregulation of PLK1 are not yet clear, although destabilization through ubiquitin-dependent degradation has been proposed (Kang, et al., 2002). Moreover, depletion of PLK1 function arrested cells with monoastral spindles, due to inhibition of centrosome maturation and separation. In contrast, mislocalized PLK1 resulting from polo box domain overexpression showed a checkpoint-dependent mitotic arrest characterized by impaired chromosome congression implying that catalytic activity of PLK1 was sufficient to bring about centrosome maturation, centrosome separation, and spindle assembly. However, complete chromosome congression and continued progression through M phase was dependent on correctly localized PLK1 activity (Hanisch, et al., 2006).

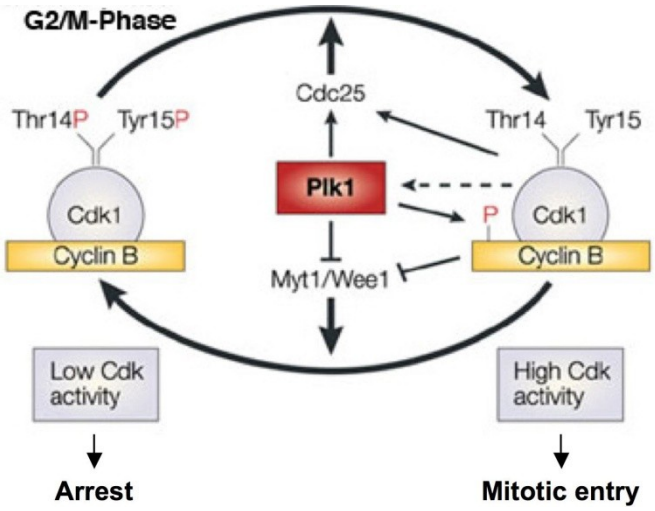

Figure 7: Complex regulatory network controls the activity of the principal mitotic regulatory kinase, cyclin-dependent kinase-1 (Cdk1). The newly formed Cdk1–cyclin-B complex is maintained in an inactive state, owing to inhibitory phosphorylation of Cdk1 at Thr14 and Tyr15 by the Wee1 and membrane-associated Cdk1-inhibitory kinase (Myt1) kinases. This inhibitory effect is overcome by dephoshporlyation of the dual-specificity phosphatase Cdc25c, which results in the activation of Cdk1–cyclin-B complex. Once activated, Cdk1 triggers a positive-feedback loop by phosphorylating Cdc25c and Wee1/Myt1, thereby causing their further activation and inhibition, respectively. PLKs also affect both Cdc25 and Myt1 (and presumably Wee1), either functioning as trigger kinases for the activation of Cdk1, or merely in the context of a positive-feedback loop. PLK1 also phosphorylates *Homo sapiens* cyclin B1 on Ser133, but the consequences of this phosphorylation are not yet known. Conversely, Cdk1–cyclin-B might contribute to PLK1 activation, most likely through indirect mechanisms (dashed arrow) (Barr, et al., 2004).

1.4 Burkitt lymphoma

Burkitt lymphoma (BL) is non-Hodgkin's lymphoma, which is an aggressive malignant B-cell tumor. There are three subtypes of BL: endemic BL, sporadic BL and HIV associated BL. Endemic BL occurs with high frequency in tropical areas of Africa and New Guinea, where it is usually associated with EBV and normally observed in children between 2-14 years. Sporadic BL occurs with lower frequency in the United States and Europe, where its EBV-association is less strong. And the third subtype, HIV associated BL, is distributed all over the world and usually associated with HIV infection (Barnett, 1968). BL is regarded as the prime example of a latency I malignancy, in which EBNA1 is exclusively expressed (A.B. Rickinson and E. Kieff, 1996). EBV latency is characterized by the expression of three different viral gene expression programs representing different levels of viral silencing (Masucci and Ernberg, 1994). Among latency III, II, I and 0, latency III is the least restrictive program and results in the expression of six EBV nuclear antigens (EBNA1-6) and three latent membrane proteins (LMP1, LMP2A, LMP2B). This expression pattern is observed in B-cells that are immortalised by EBV infection *in vitro* and is found *in vivo* in the blood and lymphoid tissues of infectious mononucleosis patients (Babcock, et al., 2000). EBNA1 is thought to be essential for the maintenance of the episomal state of EBV in infected cells and binds to the origin of replication. Latent membrane protein (LMP) is a membrane-associated protein, which is found in the virus particle as well as the infected cells. The structure of LMP resembles a growth factor receptor, which may play a role in the immortalization process. EBNA1 is oncogenic in vivo and suggest that the gene product may play a direct role in the pathogenesis of Burkitt lymphoma and possibly other EBV-associated malignancies (Wilson, et al., 1996). However, recent studies reported the identification of a subset of BL tumors that express most EBNAs except EBNA2 and the LMPs, thereby partially resembling latency III (Kelly, et al., 2002).

BL is invariably associated with chromosomal translocations, preferentially the t(8;14)(q24;q32) translocation, bringing the *c-myc* proto-oncogene in proximity with the immunoglobulin heavy chain promoter (Dalla-Favera et al., 1982; Taub et al., 1982). The proto-oncogene *c-myc* plays a pivotal role in the regulation of numerous essential cellular processes such as cell proliferation, differentiation and apoptosis (Henriksson and Luscher, 1996). The *c-myc* gene encodes the transcription factor c-Myc, which is a short-lived nuclear

phosphoprotein of the basic helix-loop-helix-leucine zipper (bHLH-LZ) class and its destruction is regulated by the ubiquitin-proteasome pathway (Bahram, et al., 2000; Bonvini, et al., 1998; Gregory and Hann, 2000). c-Myc heterodimerizes with a partner protein, named Max and regulates the transcription of specific E-box-containing genes (Grandori, et al., 1996; Henriksson and Luscher, 1996). Max also heterodimerizes with the Mad family of proteins to repress transcription and thus it antagonizes c-Myc, and promotes cellular differentiation. As a result of chromosomal translocations in Burkitt lymphomas, *c-myc* is constitutively active, which is crucial to the genesis of many cancers since it may cause gene amplification and chromosomal rearrangements (Grandori, et al., 1996; Mai, et al., 1999; Taylor and Mai, 1998). Furthermore, cells overexpressing c-Myc can bypass check points that are induced upon spindle disruption in the G2/M phase of the cell cycle (Adachi, et al., 2001). Such cells harbor secondary mutations that result in abrogation of the apoptotic response and therefore can still survive with an abnormal DNA content. Such cells harbor secondary mutations that result in abrogation of the apoptotic response and therefore can still survive with an abnormal DNA content. Particularly, loss of p53, Bax or overexpression of Bcl-x_L can cooperate with c-Myc in order to propagate polyploidy in various cell lines (Adachi, et al., 2001; Gutierrez, et al., 1999; Li and Dang, 1999; Minn, et al., 1996; Yin, et al., 1999). Additionally, cdk2 and cyclins A and B1 have been proposed as downstream effector molecules that could enable c-Myc to bypass these checkpoints (Adachi, et al., 2001; Li and Dang, 1999; Yin, et al., 1999). In line with these findings, c-Myc has been shown to disrupt signaling pathways that control cell cycle including deregulation of the cyclin/cdk2 complexes (Amati, et al., 1998). Interestingly, cdk2 activation is necessary for centrosome duplication (Tarapore, et al., 2002) and numerical as well as centrosome aberrations have been reported in many tumor types (Pihan, et al., 1998) but not in BL.

EBV infection of a *c-myc* translocation-carrying cell may provide an additional growth advantage and drives the cell further towards a fully malignant state (Bornkamm, et al., 1987). In contrast, there are speculations that Epstein-Barr virus may contribute to the deregulation of the *c-myc* gene and that this interaction may be required for tumorigenesis in the presence of some, but not all types of *c-myc* damage arising from chromosomal translocations (Ambinder and Griffin, 1991). However, it is believed that the combined effect of EBV infection and *c-myc* deregulation plays a pivotal role in BL malignancy.

1.5 Carcinogenesis

Tumor development is a multi-step process in humans. It is associated with genetic alterations, like mutations in tumor promoting (oncogenes), tumor suppressing (suppressor) genes and stability genes, which progressively drive transformation of a normal cell into a malignant form (Evan and Vousden, 2001; Evan, et al., 1992; Igney and Krammer, 2002). Thus, many cancers are age-dependent and occur later in life (Renan, 1993). Different insults, like chemical, physiological or viral agents, can induce changes in DNA that result in increased survival and proliferation. At least six important alterations have been reported to be important for tumor development including self-sufficiency in growth signals, evasion of apoptosis, insensitivity to anti-growth signals, limitless replication capacity, sustained angiogenesis and an ability to invade other tissues (Hanahan and Weinberg, 2000).

1.5.1 Treatment modalities

At present, the traditional ways to treat cancers are through surgery, chemotherapy or radiotherapy. Combinations of these techniques are often applied to achieve better results (Elshaikh, et al., 2006). Several novel therapeutics like gene therapy, immunotherapy, angiogenetic compounds and small molecular (kinase) inhibitors, are in basic and clinical development. These modalities are aiming to improve target specificity and minimise the toxic side effects on normal tissue.

1.5.1.1 Chemotherapy

In cancer therapy, chemotherapy includes all chemical drugs that are used in cancer treatment. Today, there are several different classes of anticancer substances approved for treatment, e.g. DNA-damaging agents, antimetabolites of RNA and DNA synthesis, microtubule inhibitors, nucleotide analogues and inhibitors of topoisomerases. In general, these therapeutic agents induce a cellular stress response that leads to growth inhibition and cell death. Chemotherapeutic drugs generally target fast growing cells, as they are more sensitive to DNA damage and cellular stress. Since tumor cells generally have a higher growth rate than normal cells, they are more severely affected as compared to

normal cells (Herr and Debatin, 2001). Most drugs induce activation of the intrinsic apoptotic pathway and altered functions of several Bcl-2 family proteins are important for anticancer drug resistance (Daniel, et al., 2003; Igney and Krammer, 2002). In addition, several chemical agents have also been clamied to activate the death receptors pathway (Debatin and Krammer, 2004), but the extrinsic pathway is generally not regarded as the major regulator of drug induced apoptosis in tumor cells (Kaufmann and Vaux, 2003; Klopfer, et al., 2004; Scholz, et al., 2005; Wieder, et al., 2001).

1.5.1.1.1 Taxol

Paclitaxel (Taxol), the first taxane in clinical cancer therapy, is active against a broad range of cancers that are generally considered to be refractory to conventional chemotherapy. In 1963, a crude extract from the bark of the Pacific yew *Taxus brevifolia*, a scarce and slow-growing evergreen found in the old-growth forests of the Pacific Northwest, was found in preclinical studies to have cytotoxic activity against many tumors (Wani, et al., 1971). But only in 1979 paclitaxel's unique mechanism of action as an antitumor drug was identified (Rowinsky and Donehower, 1995). The cytotoxicity of taxol is based on its ability to interfere with microtubule dynamics by binding to β-tubulin subunits in microtubules leading to inhibition of microtubule disassembly (Horwitz, 1992; Nicolaou, et al., 1994; Pazdur, et al., 1993). Microtubuli have a significant role during mitosis including the cytoskeleton break down and the mitotic spindle apparatus. Taxol operates, however, in all phases of the cell cycle. During mitosis star-shaped microtubuli formation is observed and in other cell cycle phases microtubuli bundles are formed (Horwitz, 1994; Schiff, et al., 1979). Taxol-induced apoptosis at clinically relevant doses may occur either directly after a mitotic arrest or following an aberrant mitotic exit into a G1- like multinucleated state (Abal, et al., 2001; Jordan, et al., 1993; Jordan, et al., 1996; Lin, et al., 1998; Woods, et al., 1995).

1.5.1.1.2 Vincristine and nocodazole

Vincristine belongs to the family of *vinca* alkaloids, which was isolated from the leaves of the periwinkle plant *Catharanthus roseus* (L.). Though the leaves of the periwinkle plant have been used for their medicinal properties since the seventeenth century, groups both at Eli Lilly Research Laboratories and at the University of Western Ontario discovered its cancer

chemotherapeutic potential in the late 1950s. Tubulin and microtubules (β-tubulin subunit on α/β-tubulin) are the main targets of the *Vinca* alkaloid. Functional studies showed the mitotic-blocking action of low *Vinca* alkaloid concentrations in living cancer cells and indicated that the block is due to suppression of microtubule dynamics rather than microtubule depolymerization (reviewed(Jordan and Wilson, 2004)). It is used in treatment of several cancers including leukaemias, lymphomas, breast and lung cancer. Vincristine was found to affect cells at mitotic phase and also in interphase, producing a transient G2 block at drug concentrations and drug exposure durations studied. With increasing drug exposure duration, the proportion of cells undergoing polyploidy increased progressively (Mujagic, et al., 1983). There are interesting evidences showing that knockdown of βII- or βIVb-tubulin increases sensitivity to Vinca alkaloids but not the taxanes. (Gan and Kavallaris, 2008).

Another drug, nocodazole, is similar to vincristine in its mode of action and binds to β subunit of microtubule thereby interfering with microtubule polymerization. Nocodazole induced inhibition is readily reversible (Samson, et al., 1979). Nevertheless, treatment of cells with nocodazole and other microtubule-interfering agents evokes the activation of stress response pathways, cell cycle arrest, and the induction of apoptosis. This accounts for the extensive use of microtubule-interfering agents in tumor chemotherapy.

2 Aim of the study

Burkitt lymphoma is an uncommon but highly aggressive form of non-Hodgkin's lymphoma involving B cells. The rate of cell division in Burkitt's lymphoma is one of the highest among human tumors and often life threatening. It accounts for 40-50% of childhood non-Hodgkin's lymphoma. Pediatric lymphomas are high-grade lymphomas that are diffuse and aggressive with propensitiy for wide spread dissemination. Unlike adults with NHL, who most often present with lymph-node disease, children typically have extranodal disease involving the mediastinum (26% of cases), abdomen (31%) or head and neck (29%). Pathogenesis of Burkitt's lymphoma is associated with c-Myc translocation and/or Epstein-Barr virus infection. Notebly, *PLK1* a cell cycle check point regulator gene has also begun to attract increasing attention as it is overexpressed in variety of human cancers including Burkitt lymphomas, and its expression often correlates with poor patient prognosis**.** The general objective of this thesis is to elucidate the molecular apoptosis signalling mechanisms that occur during resistance development and Burkitt lymphoma treatment in the context of mitosis regulation and ploidy control.

The specific objectives:

- To elucidate molecular mechanisms that are important for resistance development in Burkitt lymphomas against microtubule inhibitors induced apoptosis or mitotic catastrophe.
- Role of Bcl-2 family members and caspases in induction of apoptosis and polyploidy in Burkitt lymphomas.
- The contribution of cell cycle checkpoint gene *PLK1* to the development of the malignant phenotype in Burkitt lymphomas and other tumor cells.

3 Results

3.1 Inverse relationship between apoptosis and polyploidy in various Burkitt lymphoma cells upon microtubule inhibitors treatment

To understand the mechanism of cell death induced by anticancer drugs in Burkitt lymphomas, 15 different Burkitt lymphoma cell lines were investigated for sensitivity to apoptosis with microtubule inhibitors. In this approach, cells were incubated with different concentrations of taxol or nocodazole for different periods of time. Apoptosis and polyploidy were determined by flow cytometry using a modified cell cycle assay for measurement of hypodiploid and hyperploid DNA content. The range of tested concentrations was 1.0 nM to 1000 nM and time of incubation was 24 to 96 h. For example, BL2 cells showed enhanced apoptosis in response to microtubule inhibitor nocodazole and taxol, ranging up to 56% and 53%, respectively compared to <5% in control cells (Figure 8-panel A). In contrast, other cell lines like DG75, nocodazole and taxol treatment induced apoptosis up to 35% and 22%, respectively compared to <6% in control cultures in the absence of drug (Figure 8-panel B). These variations in apoptosis induction can be attributed to cell type specific phenotypes. Similiarly, all the remaining cell lines were treated and analyzed as explained above, (data not shown). Taken together the data from the investigated cell lines, it turned out that 100 nM taxol and nocodazole efficiently induced apoptosis after 72 h of treatment in many cell lines. Therefore, this concentration and time point were used as standard conditions throughout this study.

To determine the effect of taxol, a microtubule stabilizer, 15 different Burkitt lymphoma cell lines were incubated for 72 h in the presence or absence of 100 nM taxol. Hypodiploid and hyperploid DNA content was measured using flow cytometry to determine the percentage of cells undergoing apoptosis or polyploidy. Table 2 gives an overview of p53 status, Epstein Barr Virus status and translocation associated with each cell line. Taxol treatment showed apoptosis sensitivity ranging from 11% (HH514) to 69% (MutuI) as shown in Figure 9A and hyperploidy ranging from 1% (BL41 B95-8) to 58% (HH514) as shown in Figure 9B. All the cell lines left untreated exhibited less than 10% apoptosis or polyploidy. Interestingly, cell lines resistant to apoptosis displayed enhanced hyperploid DNA content,

which is considered as polyploidy and vise versa. For instance, 13% of DG75 cells displayed apoptosis compared to 45% of cells with polyploidy, whereas 50% of BL2 cells showed apoptosis compared to 3.4% cells with polyploidy. These data clearly show that taxol treated cells that are resistant to apoptosis were induced to undergo polyploidy and consequently mitotic catastrophe.

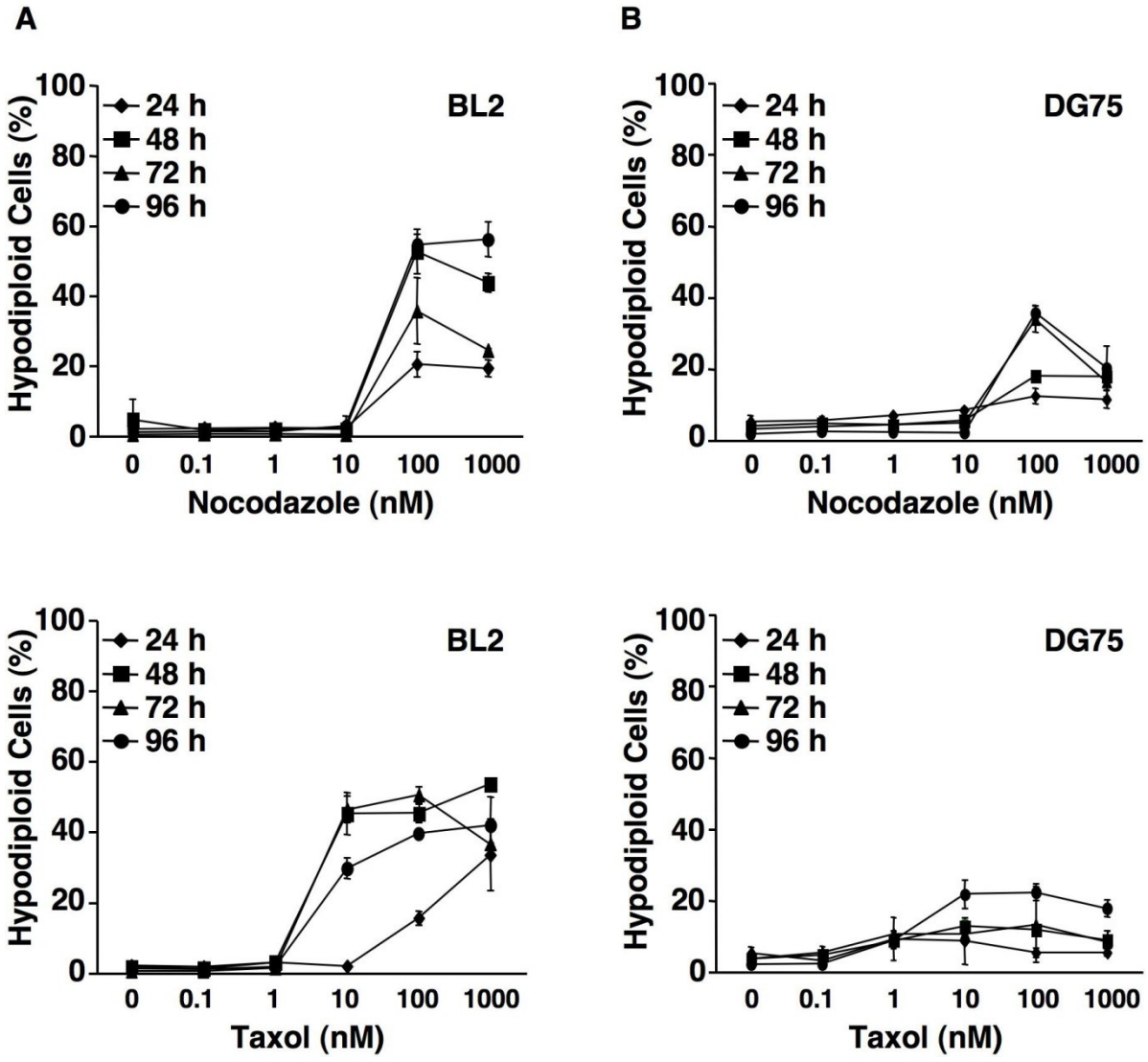

Figure 8: Microtubule inhibitors taxol and nocodazole induce apoptosis in BL2 and DG75 cells. Cells were cultured for 24 h, 48 h, 72 h and 96 h in presence or absence of taxol or nocodazole with concentration ranging from 0.1-1000 nM and subjected to flow cytometric analysis of DNA content. (A) BL2 cells with sub-G1 hypodiploid DNA content were considered apoptotic. (B) DG75 cells with sub-G1 hypodiploid DNA content were considered apoptotic. The results represent means ± SD of triplicates

Similarly, the effect of nocodazole, a microtubule destabilizer, was investigated on 15 different Burkitt lymphoma cell lines. Cells were incubated with or without nocodazole for

72 h and analyzed for the percentage of apoptotic and polyploid cells using flow cytometry for measurement of hypodiploid and hyperploid DNA content. Nocodazole treatment induced a varied percentage of apoptosis (Figure 10A) and hyperploidy (Figure 10B) in different cell lines. Similar to taxol treatment, nocodazole treatment also demonstrated enhanced polyploidy in cell lines resistant to apoptosis and vice versa. For example, 15% of CA46 cells exhibited apoptosis compared to 40% polyploidy. Whereas, 36% of BL2 cells showed apoptosis compared to 6% polyploidy, thereby confirming the inverse relationship between induction of apoptosis and polyploidy.

Table 2: Shown are the Burkitt or Burkitt like lymphoma cell lines and their EBV and c-Myc translocation status

BL Cell line	EBV status	c-Myc translocation
A1	Positive	Derived from the EREB2-5 cell line transfected with an Ig-c-myc minilocus construct and deprived of estrogen
Akata	Positive	t(8,14)
BJAB-Bclx$_L$	Negative	8q+
BJAB-WT	Negative	8q+
BL like P493-6	Positive	An EREB2-5-derivative cell line transfected with a Tet-off-inducible c-myc vector grown continuously in the absence of estrogen and tetracycline
BL2	Negative	t(8,22)
BL29	Positive	t(8;14)
BL41	Negative	t(8,14)
BL41 B95-8	Positive	t(8,14)
BL41-P3HR1	Positive	t(8,14)
BL70	Negative	t(8;14), t(12;22)
CA46	Negative	t(8;14)
DG75	Negative	t(8;14)

HH514 (Single clone of P3HR-1)	Positive	t(8,14)
Mutu1	Positive	t(8;14)

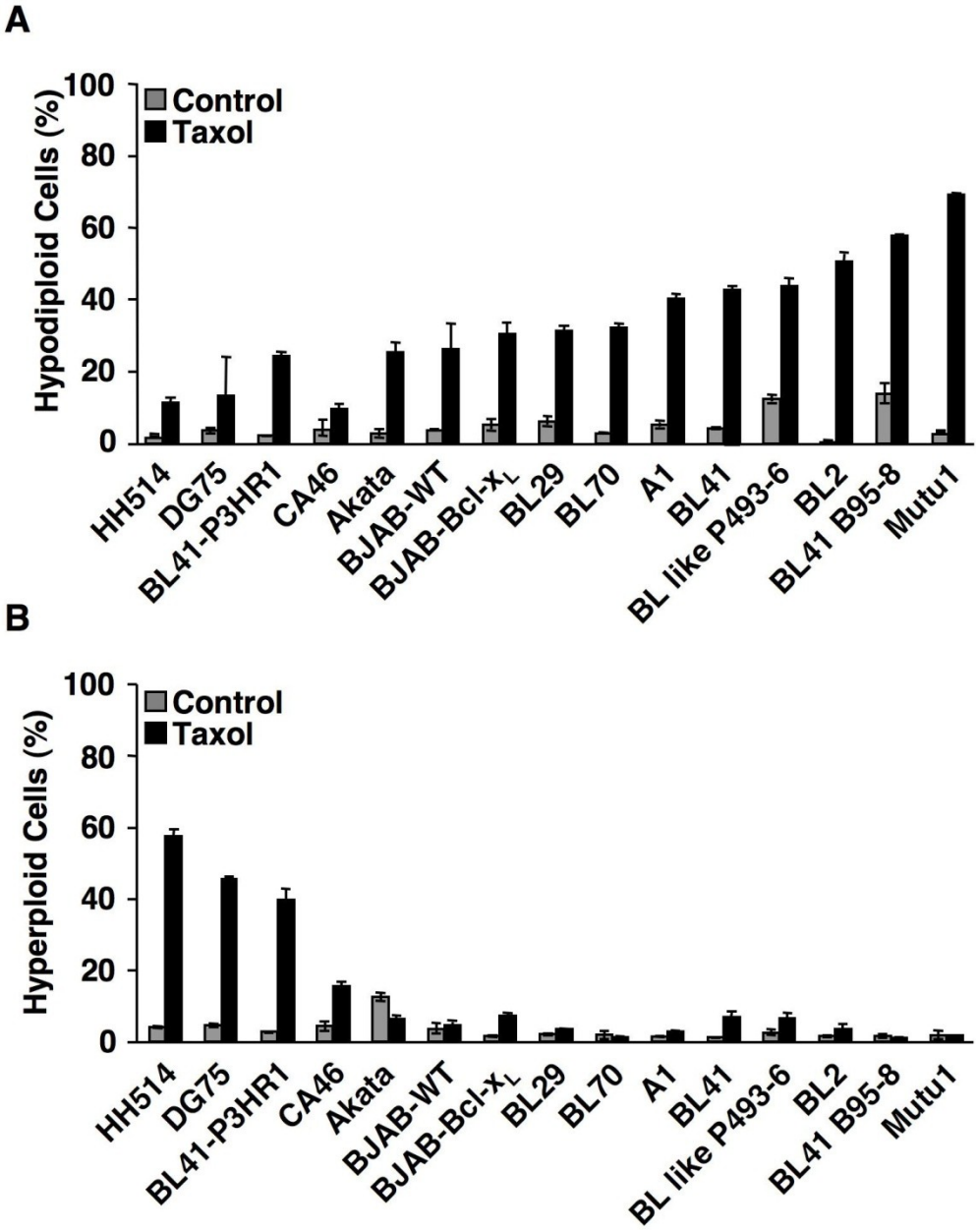

Figure 9: Taxol treated burkitt lymphoma cells show an inverse relationship between induction of apoptosis and polyploidy. 15 different Burkitt lymphoma cell lines shown in the figure were cultured for 72h in presence or absence of 100 nM taxol and subjected to flow cytometric analysis of DNA content. (A) Cells with a sub-G1 hypodiploid DNA content were considered apoptotic. (B) Cells showing hyperploid (>4N) DNA content were considered polyploid. The results represent means ± SD of triplicates

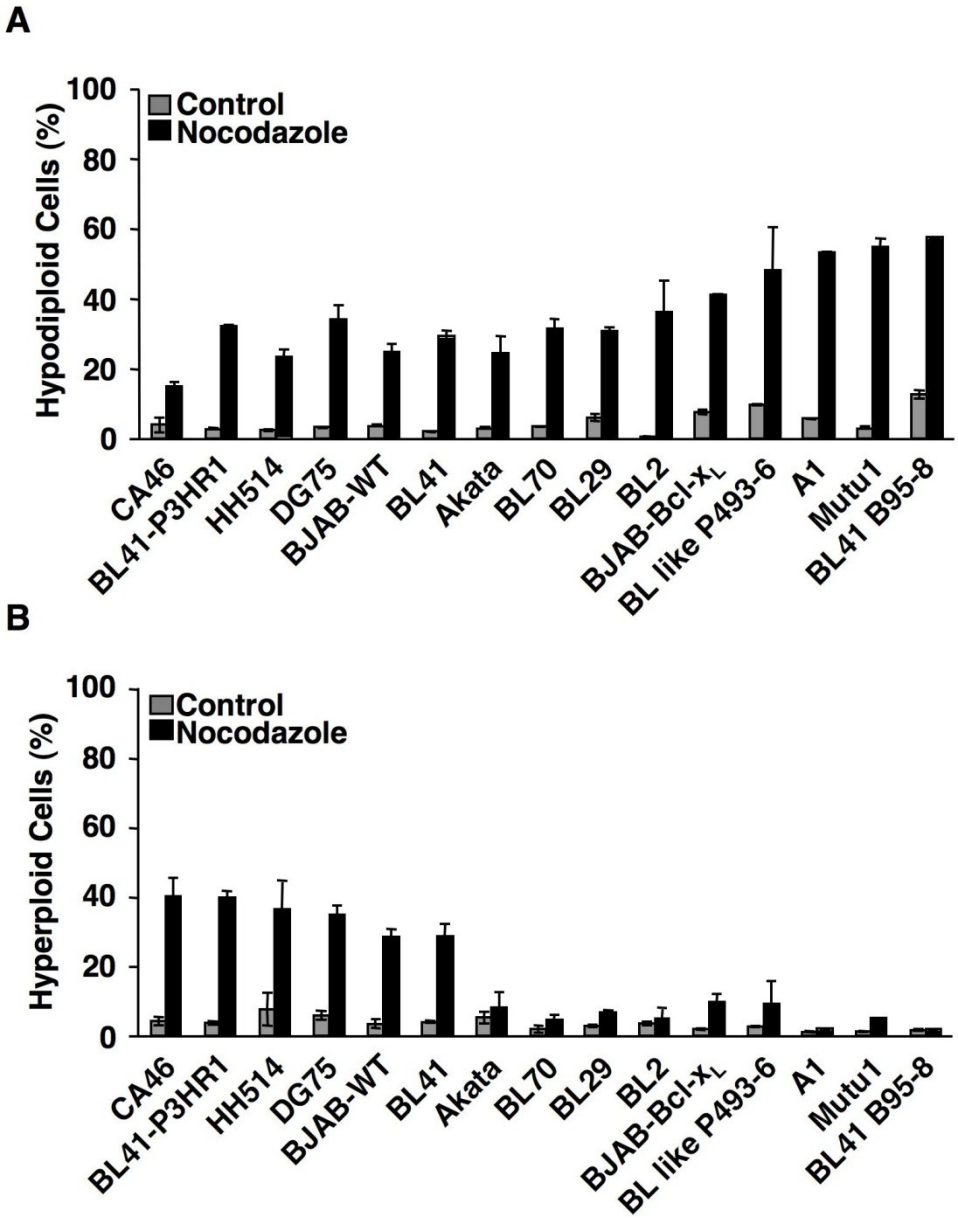

Figure 10: Nocodazole treated Burkitt lymphoma cells show an inverse relationship between induction of apoptosis and polyploidy. 15 different Burkitt lymphoma cell lines shown in the figure were cultured for 72h in presence or absence of 100 nM nocodazole and subjected to flow cytometric analysis of DNA content. (A) Cells with a sub-G1 hypodiploid DNA content were considered apoptotic (B) Cells showing showing hyperploid (>4N) DNA content were considered polyploid. The results represent means ± SD of triplicates

To further elucidate the mechanism underlying the inverse relationship between induction of apoptosis and polyploidy, four apoptotic sensitive cell lines BL2, BL70, BJAB-WT, and BL41 and three apoptosis resistant cell lines BL41-P3HR1, DG75, CA46 were chosen for

further experiments. Apoptosis sensitive cell lines are depicted throughout the thesis with an underline for better distinguishability from apoptosis resistant cells.

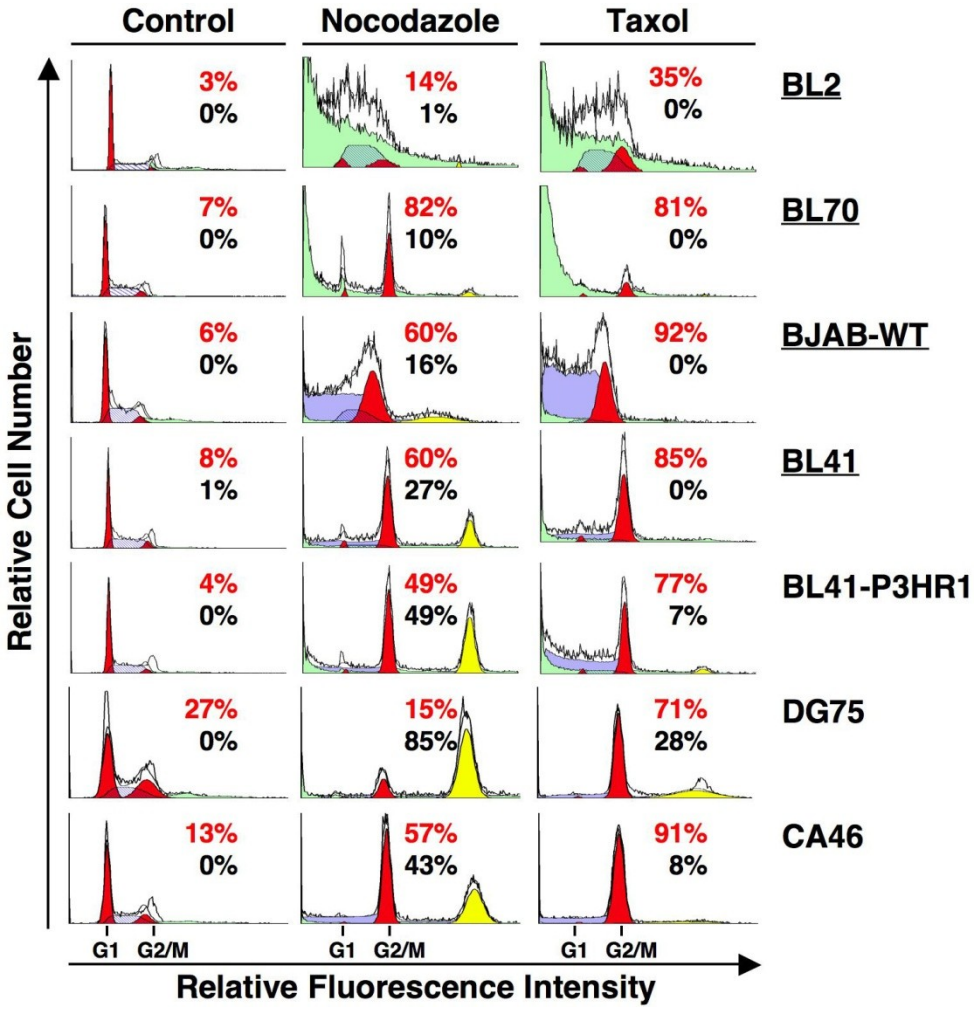

Figure 11: Taxol and nocodazole induce G2/M arrest leading to either apoptosis or polyploidy in Burkitt lymphoma cells. Data shown are from flow-cytometric analysis of cell cycle of the cells cultured for 72 h in presence or absence of 100 nM taxol or nocodazole. Linear emission of PI dye from cells was measured and percentage hyperploidy (>4N DNA content) was analyzed using MODFIT-LT software. Left red peak = G0/G1; right red peak = G2/M; hatched peak = S; Yellow peak = >4N DNA content; values in red = % of cells in G2/M (4N); values in black = % of cells with >4N DNA content.

To determine the impact of microtubule inhibitors on cell cycle, the selected 7 cell lines were incubated for 72 h in presence or absence of taxol and nocodazole respectively and assayed for cell cycle distribution and analyzed using MODFIT-LT software. As shown in Figure 11, both taxol and nocodazole induced arrest in G2-M phase of the cell cycle in apoptotic sensitive cells, compared to untreated control cells. Whereas, in apoptotic resistant cells, they induced enhanced polyploidy (>4N DNA content) in addition to G2-M cell

cycle arrest. Specifically, nocodazole treatment induced polyploidy to a greater extent. For example, up to 85% in DG75 cells compared to 28% in taxol treated cells. Although there is a difference in the extent of polyploidy induction between nocodazole and taxol treatment, both these microtubule inhibitors promote induction of polyploidy. This implies that BL cells undergo either apoptosis or polyploidy following G2/M cell cycle arrest upon treatment with microtubule inhibitors regardless of the type of inhibitor used.

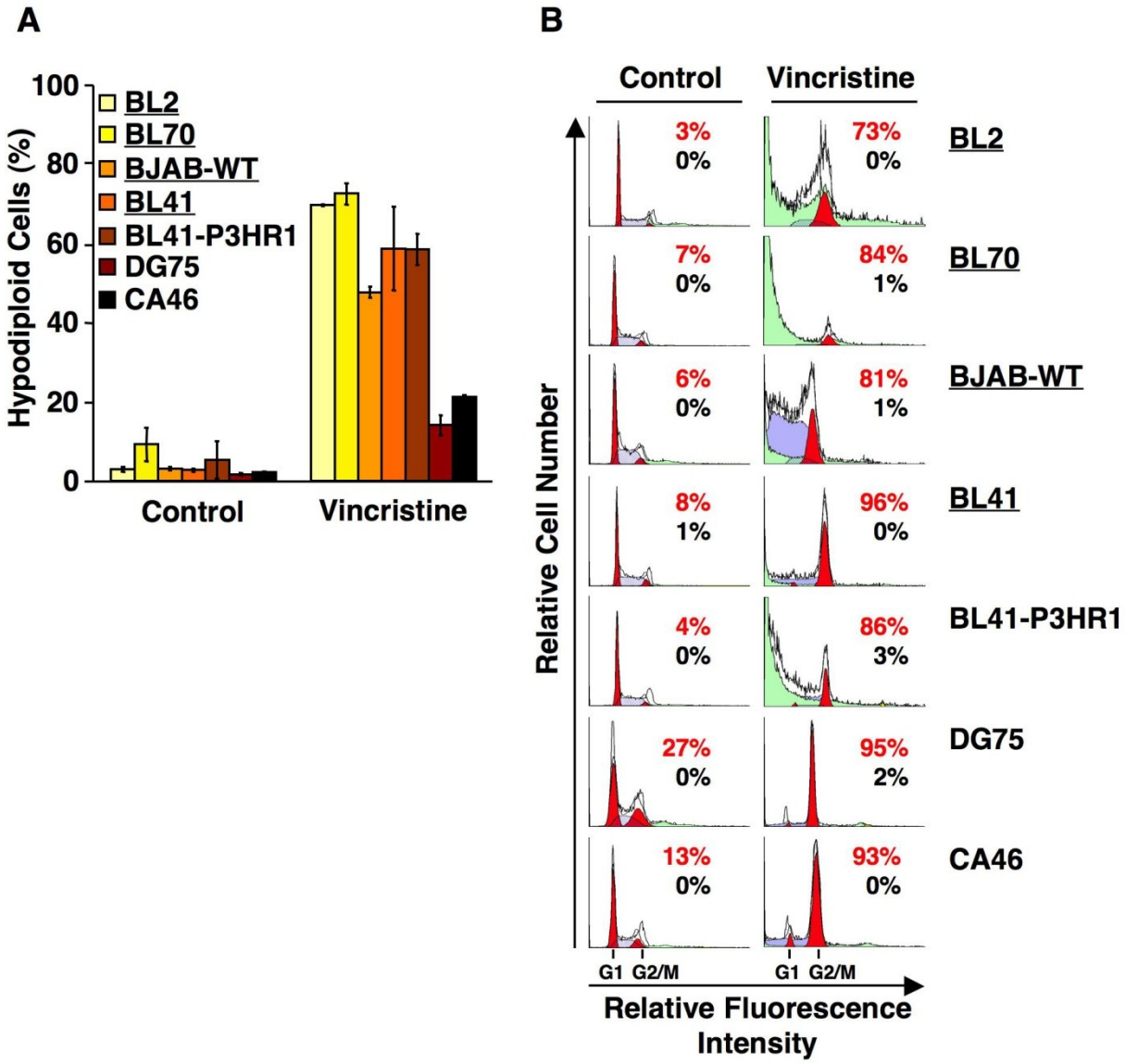

Figure 12: Vincristine induces G2/M arrest leading to apoptotic DNA fragmentation but not polyploidy in apoptotic resistant Burkitt lymphoma cells. Indicated cell lines were cultured for 72 h in presence or absence of 100 nM vincristine and subjected to flow cytometric measurement of DNA content. (A) Cells with sub-G1 hypodiploid DNA content were considered apoptotic. The results represent means ± SD of triplicates. (B)

Percentage hyperploidy (>4N DNA content) was analyzed using MODFIT-LT software. Left red peak = G0/G1; right red peak = G2/M; hatched peak = S; Yellow peak = >4N DNA content; values in red = % of cells in G2/M (4N); values in black = % of cells with >4N DNA content.

3.2 Vincristine treatment does not induce polyploidy implying different cell death mechanism compared to that of taxol and nocodazole

Vincristine a 'microtubule destabilizer' is known as a better chemotherapeutic drug for treatment of Burkitt lymphomas than taxol or nocodazole. To investigate the basis of its action, the 7 chosen Burkitt lymphoma cell lines were incubated for 72 h in the presence or absence of vincristine. Apoptosis was determined by measurement of hypodiploid DNA content using flow cytometry. Similar to taxol and nocodazole, vincristine treatment showed varied percentages of apoptosis in different cell lines ranging between 14% (DG75) and 72% (BL2) as shown in Figure 12A. All the cell lines left untreated displayed less than 10% apoptosis. In cells similarly treated, when analyzed for cell cycle distribution, the accumulation of G2/M cell population was observed. Surprisingly, apoptosis resistant cells did not show enhanced hyperploidy as shown in Figure 12B. This is in contrast to taxol or nocodazole treated cells. This implies that vincristine has a different mechanism of cell death induction as compared to taxol and nocodazole.

3.3 Microtubule inhibitors induce endoreduplication during mitosis leading to enhanced ploidy in apoptosis resistant cell lines

It is clear from the above results that microtubule inhibitors induce cell cycle arrest in the G2/M phase of the cell cycle. However, it is unclear, whether the cells are arrested in G2 or mitotic phase of cell cycle. It is possible that microtubule inhibitor treatment specifically induces aberrant mitosis in Burkitt lymphoma cells. To investigate this possibility, nocodazole, taxol or vincristine treated cells were examined by MPM2 staining against mitosis-specific phosphoepitopes and analyzed for MPM2 positive cells by flow cytometry. As shown in Figure 13A, treatment with microtubule inhibitors induced enhanced accumulation of a mitotic cell population in all cell lines. For example, up to 60% of cells showed mitotic accumulation in taxol-treated DG75 cells by 24 h. However, at this time

point there was very little effect on induction of polyploidy in most of the cell lines examined. This is similar to <10% of untreated control cells with polyploidy, except for a slight induction in DG75 cells (20%) (Figure 13B).

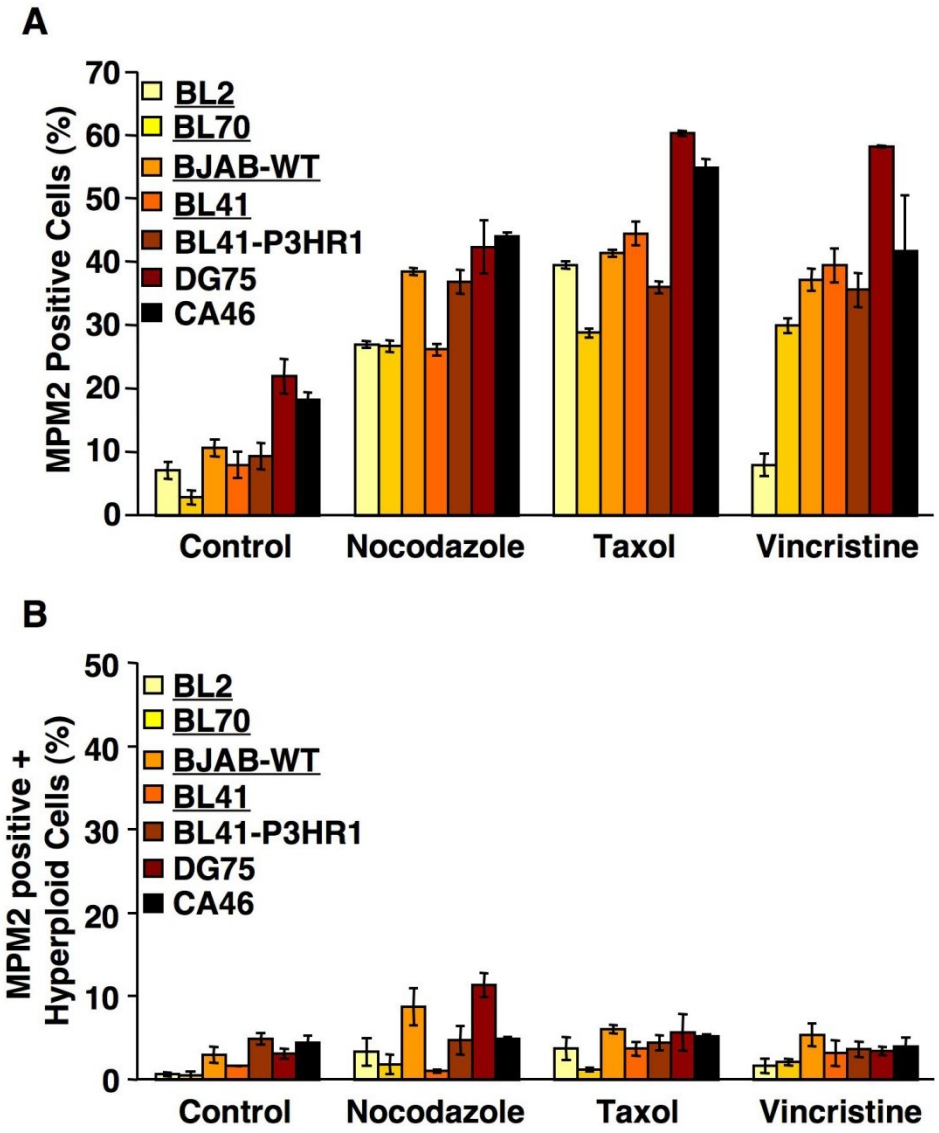

Figure 13: Microtubule inhibitors induce mitotic arrest in Burkitt lymphoma cells. Indicated cell lines were cultured for 24h in presence or absence of 100 nM nocodazole, taxol or vincristine (A) Cells were stained with a fluorescein isothiocyanate (FITC)-labeled MPM2 antibody. Bi-variate analysis of DNA content (propidium iodide) and MPM2 positive staining was performed. Data are presented as percent of mitotic cells from the total cell population. (B) Percentage of mitotic cells showing hyperploid (>4N) DNA content and were considered polyploid. The results represent means ± SD of triplicates.

Intriguingly, only apoptosis resistant cells continued to be accumulated in the mitotic phase of the cell cycle for prolonged time periods of up to 72 h. For instance, nocodazole treated DG75 cells exhibited a maximum mitotic index of up to 44% (Figure 14A). In contrast, apoptosis sensitive cell lines did not show mitotic accumulation. This is comparable to that of control or untreated cells (<10%) indicating that mitotic arrest is followed by apoptosis only in apoptosis sensitive cells.

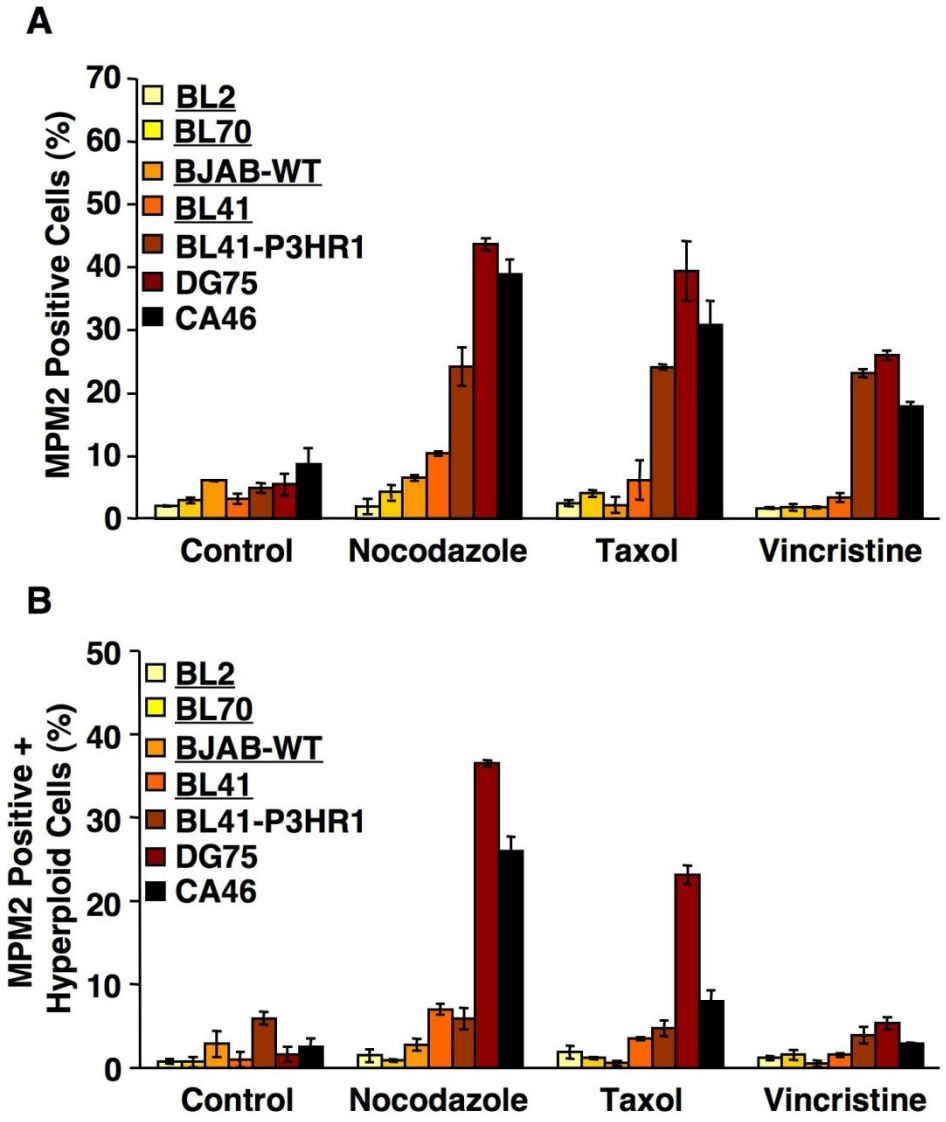

Figure 14: Microtubule inhibitors induce prolonged mitotic arrest and trigger polyploidy in apoptosis resistant Burkitt lymphoma cells. Indicated cell lines were cultured for 72 h in presence or absence of 100 nM nocodazole, taxol or vincristine (A) Cells were stained with a fluorescein isothiocyanate (FITC)-labeled MPM2 antibody. Bi-variate analysis of DNA content (propidium iodide) and MPM2 positive staining was performed. Data represented as percent of mitotic cells from the total cell population. (B) MPM2 positive cells showing hyperploid (>4N) DNA content and were considered polyploid. The results represent means ± SD of triplicates.

Since the apoptosis resistant cells exhibited enhanced polyploidy after nocodazole or taxol treatment, it's befitting to verify if the enhanced ploidy is a result of endoreduplication, a mechanism involving chromosomal DNA replication without intervening mitosis or cytokinesis, leading to an increase in the ploidy level. In this regard, positive cells for MPM2, an antibody recognising mitotic phosphoepitopes, were gated and cells exhibiting polyploid DNA content were analyzed by flow cytometry. Most of the cells positive for MPM2 (Figure 14A) displayed polyploid DNA content (Figure 14B). For instance, nocodazole treatment showed 43% of DG75 cells in mitotic phase. Interestingly, 37% of nocodazole treated DG75 cells showed both MPM2 positive and hyperdiploid DNA content. These data suggests that, cells resistant to the microtubule inhibitors nocodazole and taxol undergo endoreduplication leading to an increase in ploidy level and this event of endoreduplication is happening at the M phase of cell cycle.

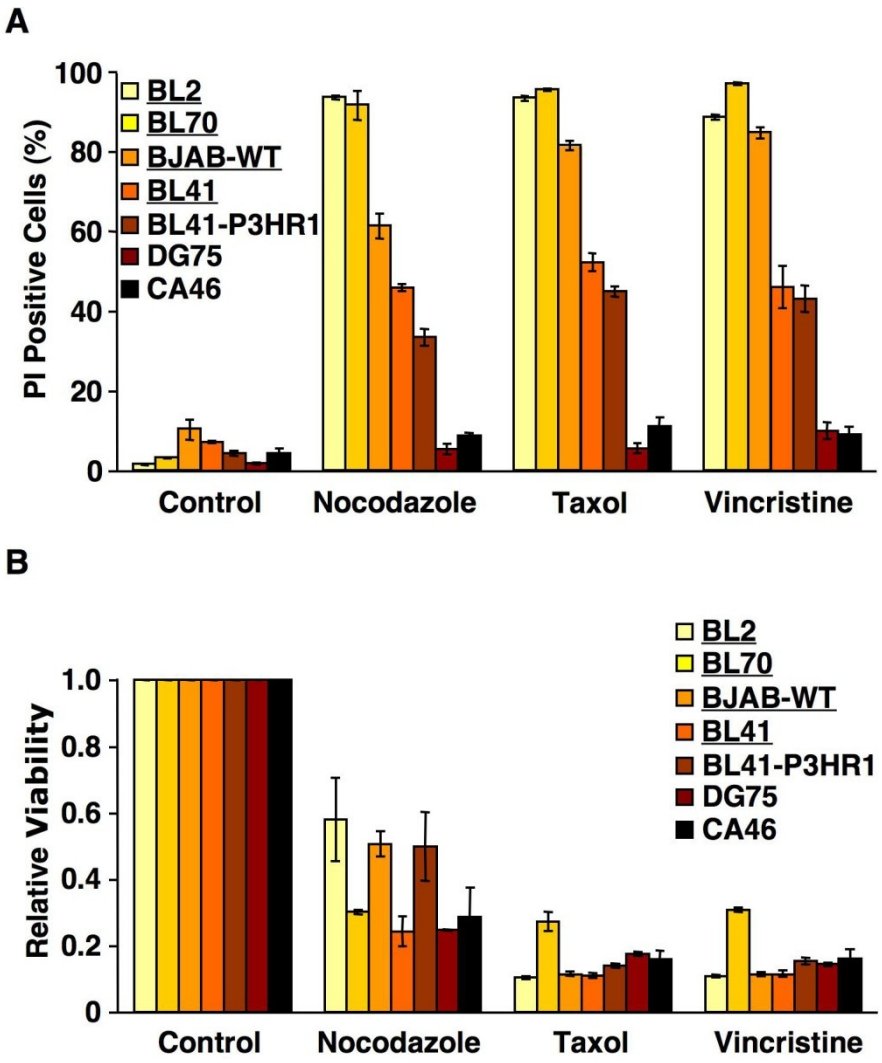

Figure 15: Loss of cell viability is not associated with immediate cell death in microtubule inhibitors induced apoptosis resistant Burkitt lymphoma cells. Cells were cultured for 72 h in presence or absence of 100 nM nocodazole, taxol or vincristine (A) Cell death measurement by PI uptake. Cells were stained with PI and% PI positive (dead) cells were measured by flow cytometry. (B) Cell viability measurement by colorimetric XTT assay. The viability of treated cells is expressed relative to that of untreated cells, which were set to 1. The results represent means ± SD of triplicates.

However with vincristine treatment, apoptosis resistant cells showed prolonged mitotic arrest for up to 72 h but displayed no sign of enhanced polyploid DNA content in mitotic cell population compared to control cells. This suggests that vincristine fails to induce endoreduplication and this is in contrast to nocodazole and taxol.

3.4 Mitotic catastrophe is not associated with immediate cell death

To verify if cells resistant to microtubule inhibitors would undergo other means of cell death or growth inhibition, cells were incubated in the presence or absence of nocodazole, taxol or vincristine for 72 h. Induction of cell death was confirmed by propidium iodide (PI) uptake by flow cytometric analysis. As shown in Figure 15A, apoptosis sensitive cells showed enhanced PI uptake. Microtubule inhibitors induced PI uptake ranging between 46% and 97%, representing the dying cells, compared to < 10% in untreated cells. In contrast, there was no enhanced PI uptake in apoptosis resistant cells upon microtubule inhibitor treatment, compared to that of untreated cells. To check the proliferative ability after microtubule inhibitor treatment, cells were subjected to a XTT cell proliferation assay and analyzed for relative cell viability. Untreated control cells were normalized to 1. Highly decreased cell viability was observed in all cell lines treated with microtubule inhibitors, irrespective of their sensitivity (Figure 15B), suggesting a prominent loss of cell proliferative ability in presence of microtubule inhibitors in all cell lines tested.

3.5 Taxol induced loss of viability is irreversible

To investigate if microtubule inhibitor induced loss of viability can be rescued, cells were incubated in the presence or absence of taxol. After 72 h of incubation the cells were washed and resuspended in a drug free medium for another 72 h and analyzed for cell viability by XTT cell proliferation assay. Taxol treated cell population demonstrated reduction in viability relative to that of cells untreated for 72 h, which was set to 1. Interestingly, cells that were rescued from taxol treatment did not show restoration of viability (Figure 16A). To confirm the above result, cells were treated in a similar way and analyzed for cell viability by trypan blue exclusion. As shown in Figure 16B, treated cells failed to recover and proliferate even after reculturing them in taxol free medium for 72 h, thereby implying that the effect of taxol on Burkitt lymphomas is irreversible, at least on a population level.

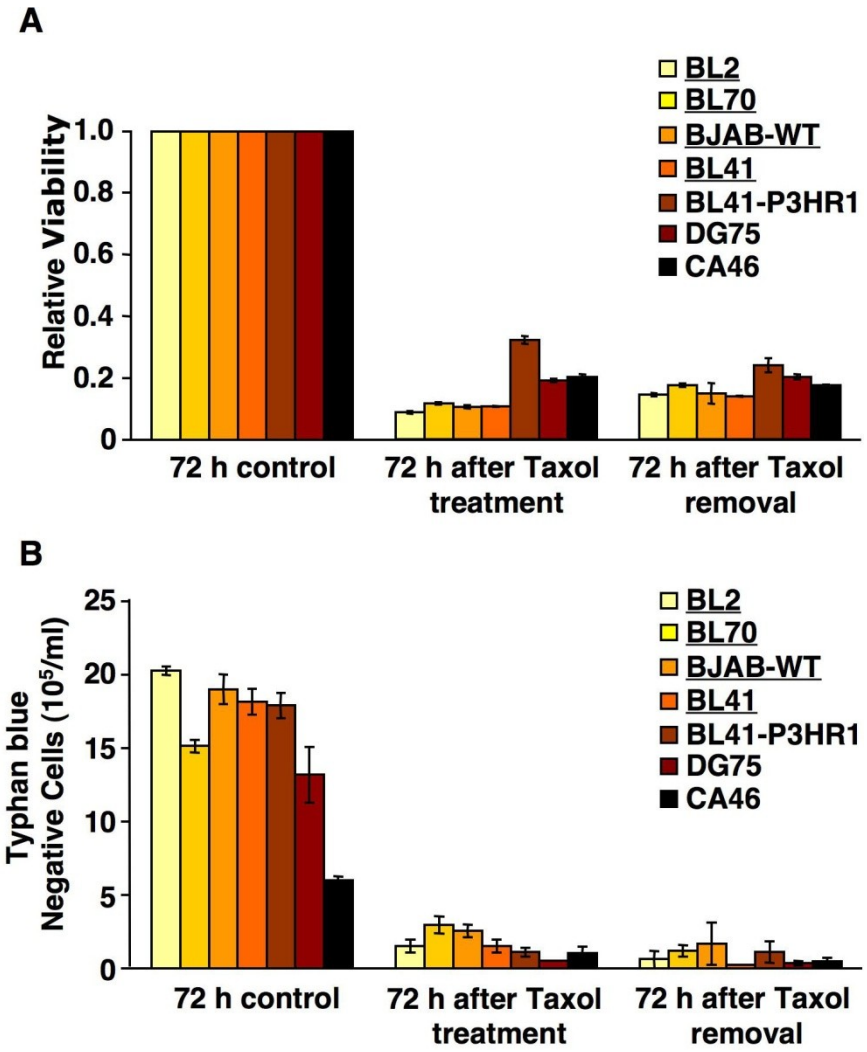

Figure 16: Loss of cell viability in Burkitt lymphoma cells is irreversible. Cells were cultured for 72h in presence or absence of 100 nM taxol. Cells were then washed and cultured in medium without taxol for an additional 72 h. (A) Cell viability was determined by colorimetric XTT assay. The viability of treated cells was expressed relative to that of untreated cells cultured, which were set to 1. (B) Cell viability was also determined by trypan blue exclusion assay. The results represent means ± SD of triplicates.

3.6 Taxol induced caspase activation

To unravel the apoptotic signalling pathway triggered by microtubule inhibitors, cells were treated with taxol or left untreated (control) for 24 h and 48 h time periods. Western blot analysis was performed to assess processing of individual procaspases (9, 3, and 8), and the caspases-3 and -8 substrate Bid, a known activator of mitochondrial apoptosis. Taxol induced caspases-9, -3, and -8 processing by 24 h (Figure 17A) and even more by 48 h (Figure 17B) in apoptosis sensitive cell lines. Cleavage of caspases-9, -3, and -8 was inhibited in cells

resistant to taxol-induced apoptosis, which may be interpreted as a lack of signals for caspase processing as measured by western blot analysis. Caspase processing was paralleled by a reduction in Bid protein levels after exposure to taxol. However this reduction in the Bid proform upon taxol treatment was observed in both apoptosis sensitive and resistance cell lines. This indicates that Bid processing is not associated with apoptosis resistance or polyploidy induction.

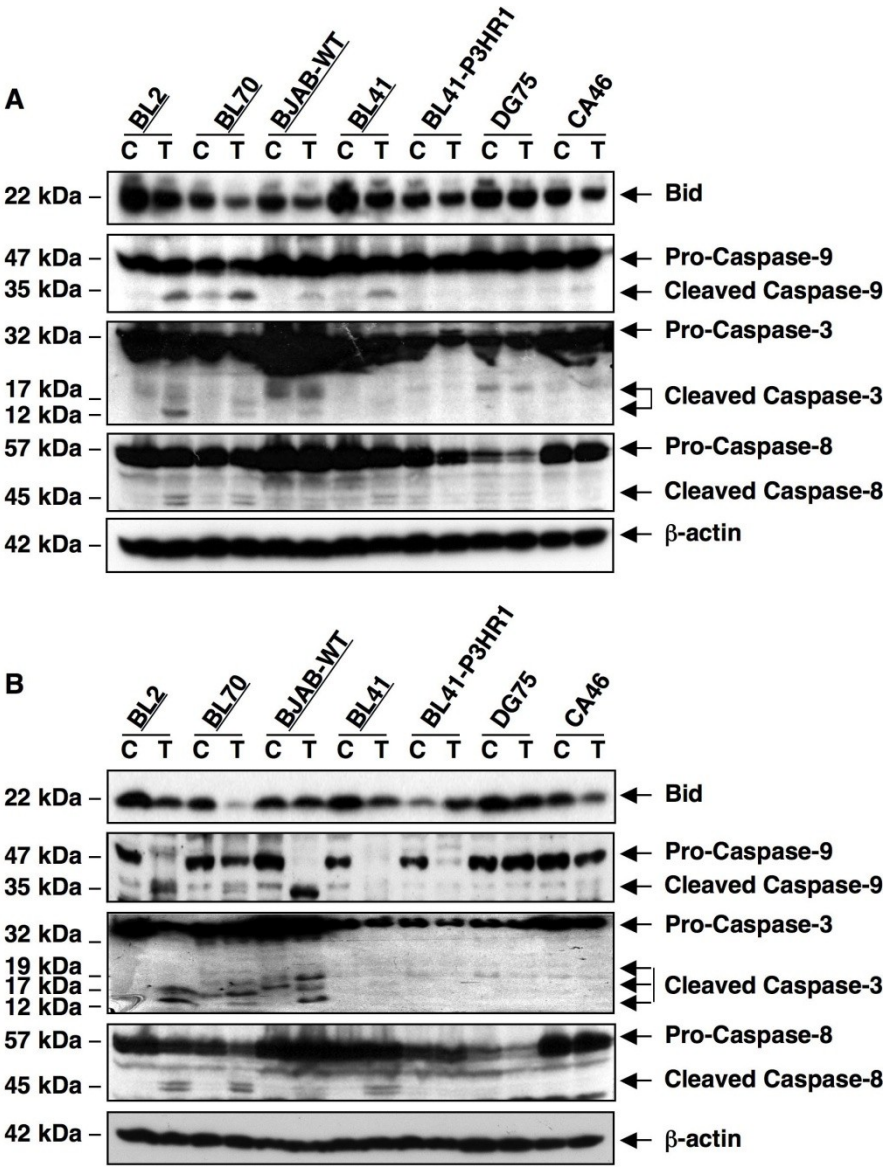

Figure 17: Taxol induces caspase processing in apoptosis sensitive but not in resistant Burkitt lymphoma cells. Indicated cell lines were cultured in presence or absence of 100 nM taxol and lysates were subjected to immunoblot analysis for Bid, and cleavage of caspase-9, -3 and -8. (A) 24 h treated cells (B) 48 h treated cells.

Molecular masses are indicated at the left. C = control or untreated cells and T= taxol treated cells. β-actin was used as a loading control.

Detection of caspase-9 p35 subunit or caspase-3 p17 and p12 subunits indicates cleavage of the zymogen to their active form. Further, caspase-9 and -3 activity was confirmed by flow cytometric analysis. To study this, the substrates FAM-LEHD-FMK and FAM-DEVD-FMK were used to assay caspase-9- and -3-like activity, respectively, in cells treated with nocodazole, taxol or vincristine for 48 h. Caspase-9 (Figure 18A) and caspase-3 activity (Figure 18B) were detected. This confirmed the previous observations that taxol induces apoptosis through caspase activation in apoptosis sensitive cell lines but not in resistant cell lines.

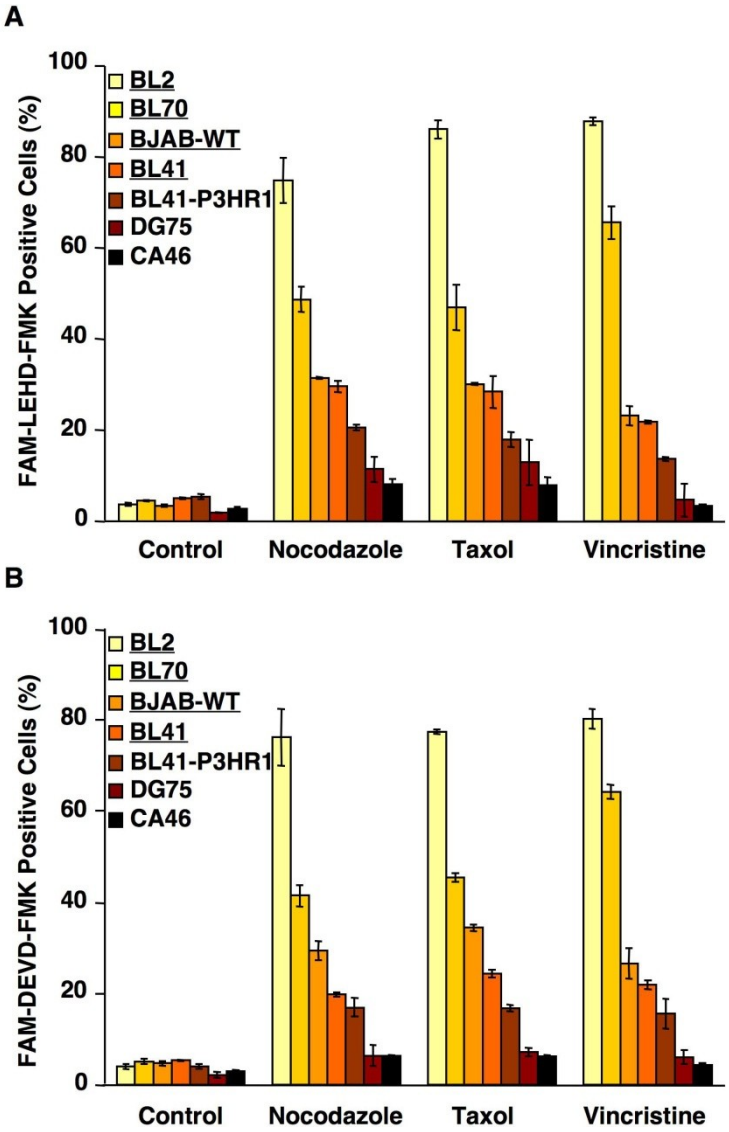

Figure 18: Taxol induces caspase like activity in apoptotic sensitive but not in resistant Burkitt lymphoma cells. Flow cytometric analysis of cells with increased Caspase-9- and -3-like activity after culturing for 48 h in presence or absence of 100 nM taxol, nocodazole or vincristine, respectively (A) Caspase-9-like activity using

FAM-LEHD-FMK substrate (B) Caspase-3-like activity using FAM-DEVD-FMK substrate. The results represent means ± SD of triplicates.

3.7 Taxol induced cell death is p53 independent

Exposure to cellular stress can trigger the p53 tumor suppressor, a sequence-specific transcription factor, to induce cell growth arrest and apoptosis. It is known that cell death induced through the p53 pathway is executed by the caspase proteases, which, by cleaving their substrates, lead to the characteristic apoptotic phenotype. In connection to earlier data showing caspase activation in apoptotic sensitive cell lines, it is befitting to check for p53 in taxol induced apoptosis and polyploidy.

Table 3: Showing the EBV, p53 and type of p53 mutations associated with each cell line. WT: Wild type; (SM): Single mutation; (DM): Double mutation; (-): Negative; (+): Positive

Burkitt Lymphoma	EBV	p53	Mutation Sequence
BL2	-	WT	
BL70	-	Mutant	R273C (SM)
Bjab WT	-	Mutant	H193R (SM)
BL41	-	Mutant	R248Q (SM)
BL41-P3HR1	+	Mutant	Y163H, E287Stop (DM)
DG75 (B Cell Line)	-	Mutant	R283H (SM)
CA46	-	Mutant	R248Q (SM)

Table 3 gives an overview of p53 status, type of p53 mutations and Epstein Barr Virus status associated with each cell line. Cells were treated (T) or left untreated (C) with taxol for 24 h and 48 h and the cell lysates were analyzed for p53 by immunoblotting. Wildtype p53 is undetectable due to its rapid turnover in basal conditions and activation of p53 or mutation(s) in p53 involve its stabilization, which can be detected by immunoblotting. Apoptosis sensitive cell line BL2, having wild type p53 did not show stabilization of p53 after taxol treatment (Figure 19A). A similar response was observed as well at 48 h after taxol treatment (Figure 19B). This indicated that p53 is not involved in taxol-induced apoptosis. Moreover, cell lines with mutated p53 displayed distinct behaviour either exhibiting

sensitivity or resistance to taxol induced apoptosis. This clearly indicated lack of correlation between p53 status and taxol induced apoptosis or polyploidy in Burkitt lymphomas.

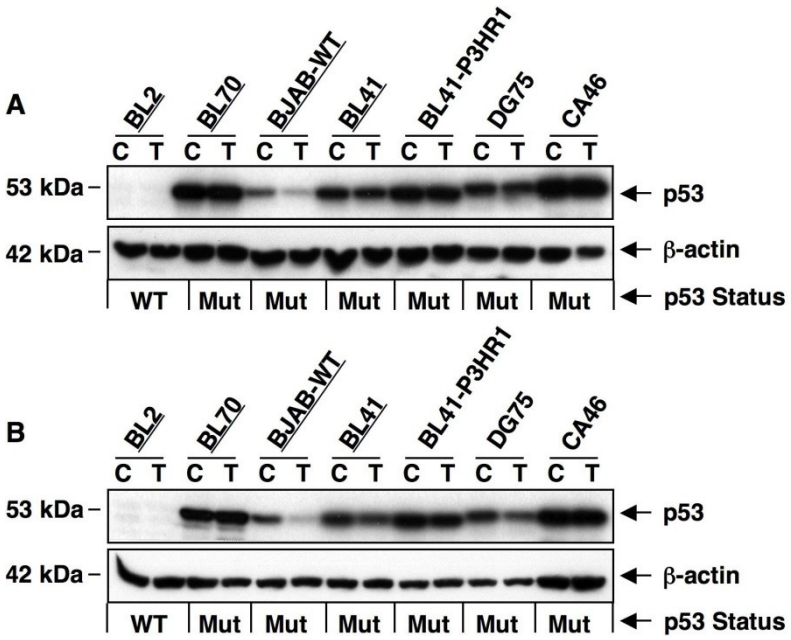

Figure 19: Regulation of p53 in Burkitt lymphoma cells upon taxol treatment. Indicated cells lines were cultured in presence or absence of 100 nM taxol and lysates were subjected to immunoblot analysis for p53. (A) 24 h treated cells (B) 48 h treated cells. C = control or untreated cells and T= taxol treated cells. β-actin was used as a loading control.

3.8 Lack of Bax and Bak can be the cause of apoptosis resistance to taxol in Burkitt lymphomas

Bax and Bak constitute an essential apoptosis gateway specifically operating at the mitochondria to regulate the intrinsic apoptotic pathway. To further investigate the role of Bax and Bak behind these discrete phenotypes (Apoptosis sensitivity and resistance) to taxol, cells were treated with taxol (T) or left untreated (C) for 24 h and 48 h. The cell lysates were analyzed for Bak and Bax protein expression by immunoblotting. As shown in Figure 20A and B, cell lines showing induction of apoptosis to taxol treatment are Bax and Bak proficient, whereas cell lines highly resistant to apoptosis induced by taxol treatment lack Bax and have very low Bak protein expression levels. However, BL41-P3HR1 cell line is an exception showing Bax and Bak proficiency. This can be justified by its EBV positive status carrying latent P3HR1 virus (and express only EBNA1), which is associated with conferring apoptosis

resistance and genomic instability. Bax protein level was reduced upon treatment with taxol, possibly due to proteolytic cleavage, which is known to enhance cell death function at the mitochondria. In conclusion, Bax and Bak appear to be involved in taxol-induced apoptosis since a loss or reduction of Bax or Bak in DG75 and Ca45 cells seems to be associated with apoptosis resistance and enhanced polyploidy.

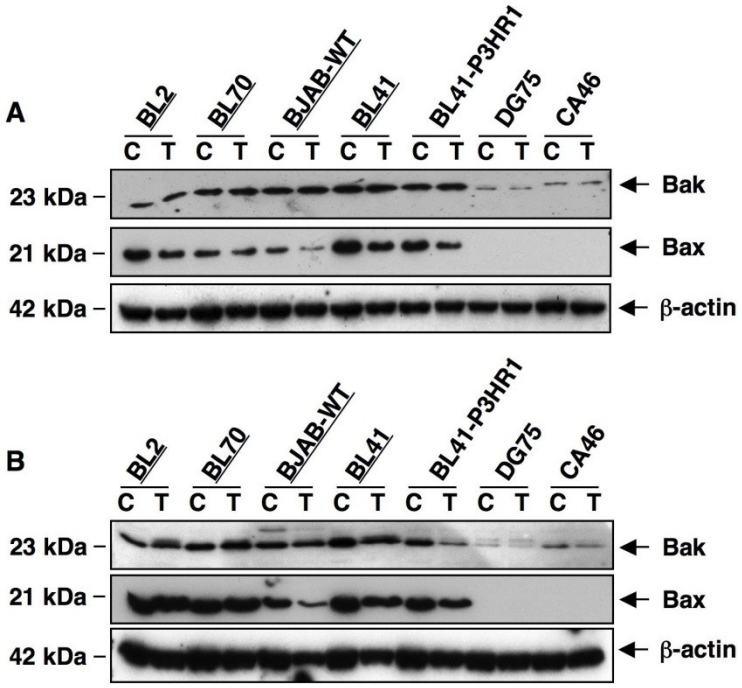

Figure 20: Apoptosis resistance Burkitt lymphomas DG75 and CA46 lack Bax and show low Bak protein expression. Indicated cell lines were cultured in presence or absence of 100 nM taxol and lysates were subjected to immunoblot analysis for Bax and Bak. (A) 24 h treated cells (B) 48 h treated cells. Molecular masses are indicated at the left. C = control or untreated cells and T= taxol treated cells. β-actin was used as a loading control.

3.9 Role of pro-apoptotic BH3-only proteins in taxol induced cell death

Since Bax and Bak seemed to be crucial regulators of taxol mediated cell death, deciphering the regulation of BH3-only proteins, (which play a key role in sensing apoptotic stimuli and initiating apoptosis by activation of Bax or Bak) may provide the basis of taxol mediated cell death. To investigate the role of BH3-only proteins, cells were treated (T) or left untreated (C) with taxol for 24 h and 48 h and cell lysates were analyzed for BH3-only proteins Noxa, Bim, Puma and Nbk by immunoblotting. Surprisingly, most of the BH3 protein

expression levels analyzed decreased after treatment with taxol by 24 h time point except Puma, which was differently regulated depending on the cell line (Figure 21A). Further, similar response was shown at 48 h time point (Figure 21B). Downregulation of BH3-only proteins might be a survival mechanism of cells to counteract taxol-induced apoptosis.

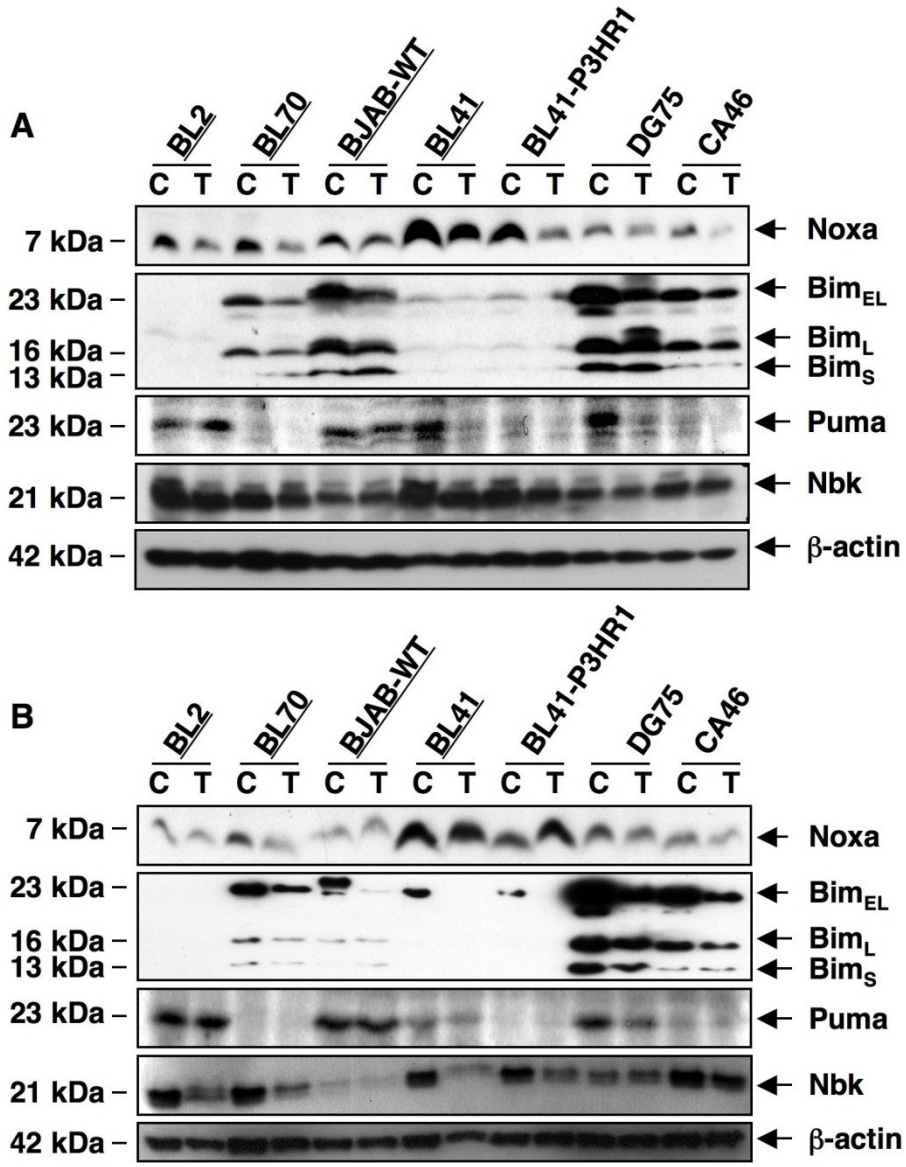

Figure 21: Taxol induces downregulation of proapoptotic BH3-only proteins in Burkitt lymphoma cells. Indicated cell lines were cultured in presence or absence of 100 nM taxol and lysates were subjected to immunoblot analysis of Noxa, Bim, Puma and Nbk (A) 24 h treated cells (B) 48 h treated cells. Molecular masses are indicated at the left. C = control or untreated cells and T= taxol treated cells. β-actin was used as a loading control.

3.10 Taxol induces downregulation of the anti-apoptotic Bcl-2 family protein Mcl-1

Since BH3-only proteins are not regulated in taxol-induced apoptosis, it is interesting to examine whether modulation of pro-survival proteins is involved in taxol-induced cellular death. In this regard, cells were treated with taxol (T) or left untreated (C) for 24 h and 48 h and cell lysates were analyzed for antiapoptotic proteins Bcl-x_L and Mcl-1 expression by immunoblotting. Most of the cell lines showed no effect on Bcl-x_L expression upon taxol treatment except for BL41 and CA46 cell lines with a slight downregulation, which might be a cell type specific consequence. In contrast, the Mcl-1 protein level was decreased by 24 h (Figure 22A) and grossly reduced by 48 h (Figure 22B) after treatment with taxol. These results suggest that taxol induces downregulation of Mcl-1, thereby disengaging the pro-apoptotic proteins t-Bid and Bak to execute apoptosis. However, dowregulation of Mcl-1 is observed in both apoptosis sensitive and resistant cell lines, indicating that Mcl-1 is not involved in apoptosis resistance and polyploidy induction.

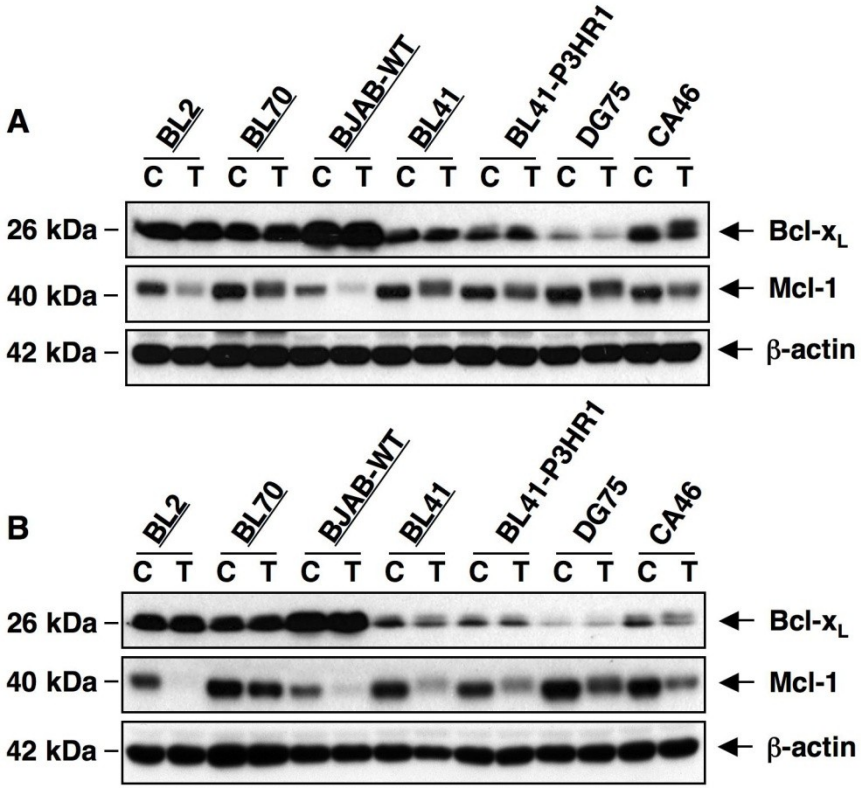

Figure 22: Taxol induces down-regulation of the anti-apoptotic BH3 only protein Mcl-1 in Burkitt lymphoma cells. Indicated cell lines were cultured in the presence or absence of 100 nM taxol and lysates were subjected

to immunoblot analysis of Bcl-x_L and Mcl-1 (A) 24 h treated cells (B) 48 h treated cells. Molecular masses are indicated at the left. C = control or untreated cells and T= taxol treated cells. β-actin was used as a loading control.

Taken together, these results indicated that taxol activates profound mitochondria-dependent death events by 24 h - 48 h. Upstream apoptotic regulators Bid and Mcl-1 seem to be regulated in both apoptosis resistant and sensitive cell lines, indicating that they are important for apoptosis induction but not for conferring resistance and induction of polyploidy. However, Bax and Bak play an important role in apoptosis resistance as taxol failed to induce caspase activation in cell lines without Bax and with weak expression of Bak.

3.11 Taxol induced apoptosis is Bax and Bak dependent

It is evident from earlier data that lack of Bax and Bak leads to apoptosis resistance upon taxol treatment. To confirm whether taxol induced apoptosis exclusively depends on Bax and Bak proficiency and represents the deciding factor for polyploidy induction, FDM-WT, FDM-*bax*$^{-/-}$ and FDM-*bak*$^{-/-}$ and FDM *bax/bak*$^{-/-}$ cells, respectively were incubated with different concentrations of taxol ranging from 1-100 nM for 24 h to 96 h. Hypodiploid DNA content was measured by flow cytometry to determine apoptosis induction. Taxol treatment resulted in enhanced apoptosis reaching up to 65% in FDM-WT cells (Figure 23A) by 96 h. There was little inhibition of apoptosis reaching up to 54% and 46% in FDM-*bax*$^{-/-}$ and FDM-*bak*$^{-/-}$ cells respectively, proving their functional redundancy (Figure 23C-D). In contrast, *bax/bak* double knock out FDM cells were highly resistant to apoptosis, irrespective of taxol concentration and duration of treatment (Figure 23B).

Treatment of cells with 100 nM taxol for 72 h was chosen to further analyze the correlation between induction of apoptosis and polyploidy using flow cytometry. FDM-WT, FDM-*bax*$^{-/-}$ or FDM-*bak*$^{-/-}$ cells exhibited enhanced apoptosis (upto 55% of cells) as shown in Figure 24A, but not polyploidy (<8% of cells) upon taxol treatment (Figure 24B). In contrast, FDM *bax/bak*$^{-/-}$ cells were highly resistance to apoptosis, with less than 5% of cells undergoing apoptosis. Interestingly, 28% of FDM *bax/bak*$^{-/-}$ cells showed enhanced polyploidy (Figure 24A-B). Thereby, confirming the inverse correlation between apoptosis and polyploidy induction, which was observed earlier in Burkitt lymphoma cells.

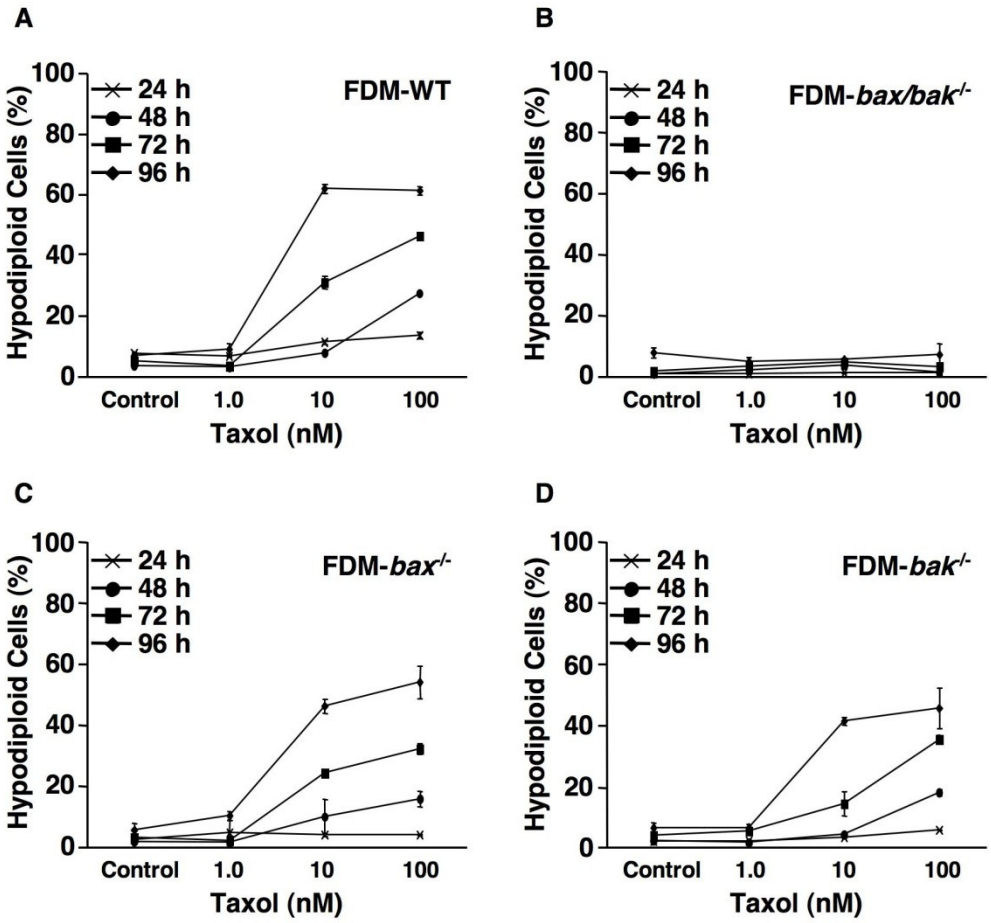

Figure 23: Taxol induces apoptosis in WT, *bax* or *bak* deficient but not in *bax/bak* double deficient FDM cells. FDM-WT, FDM-*bax*$^{-/-}$, FDM- *bak*$^{-/-}$, FDM-*bax/bak*$^{-/-}$ cells were cultured with different concentrations of taxol for24 - 96 h as depicted and subjected to flow cytometric analysis of DNA content. Cells with a sub-G1 hypodiploid DNA content were considered apoptotic. (A) Taxol-induced apoptosis in FDM-WT cells (B) Taxol-induced apoptosis in FDM- *bak/bax*$^{-/-}$ (C) Taxol-induced apoptosis in FDM-bax$^{-/-}$ (D) Taxol-induced apoptosis in FDM-*bak*$^{-/-}$. The results represent means ± SD of triplicates.

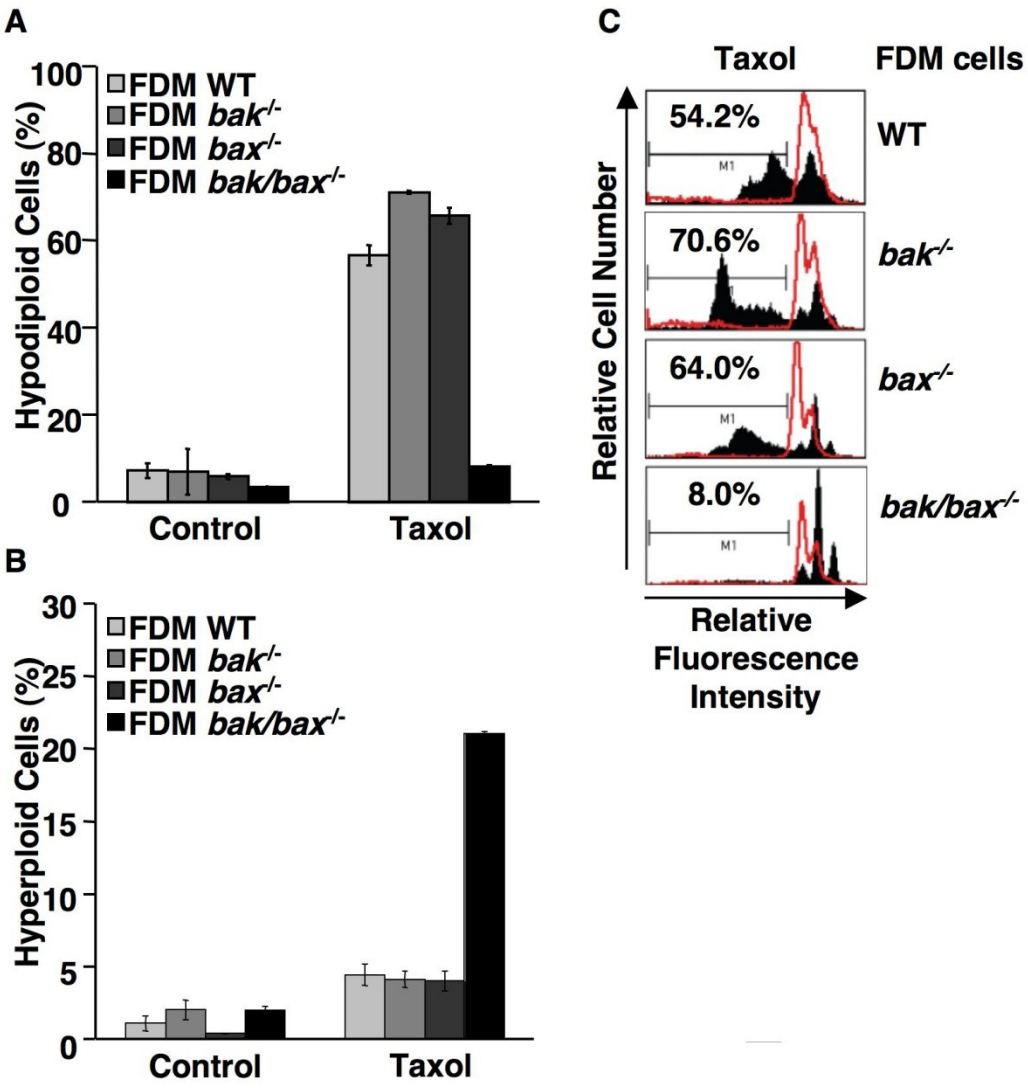

Figure 24: Taxol induces apoptosis in WT, *bax* or *bak* deficient but leads to polyploidy in bax/bak double knockout FDM cells. FDM-WT, FDM-*bax*$^{-/-}$, FDM-*bak*$^{-/-}$, FDM-*bax/bak*$^{-/-}$ cells were cultured for 72 h in the presence or absence of 100 nM taxol and subjected to flow cytometric measurement of DNA content. (A) Cells with a sub-G1 hypodiploid DNA content were considered apoptotic. (B) Cells showing hyperploid (>4N) DNA content were considered polyploid. (C) Representative DNA histograms for 100 nM taxol-induced genomic DNA fragmentation and polyploidy histograms (in black) overlayed with control histograms (in red). Percentages of apoptotic cells are given in the histograms for taxol treated cells. The results represent means ± SD of triplicates.

3.12 Individual pro-apoptotic BH3-only proteins have no impact on taxol induced apoptosis in FDM cells

To investigate whether the loss of function of various pro-apoptotic BH3-only proteins in FDM cells have an impact on taxol induced cell death, FDM cells with knockout of *puma*, *bim*, *bid* and *noxa*, respectively were incubated with different concentrations of taxol ranging from 0 to 100 nM for 72 h and 96 h. Apoptosis was determined by flow cytometry using a modified cell cycle assay for measurement of hypodiploid DNA content. Taxol treatment induced apoptosis reaching up to 65% in FDM-WT cells (Figure 23A) at 96 h. But none of the taxol treated proapoptotic BH3-only protein knockout cells showed a decrease in apoptosis induced by taxol treatment (Figure 25A-D). These results suggest that individual knock out of pro-apoptotic BH3-only proteins does not confer protection against taxol induced apoptosis.

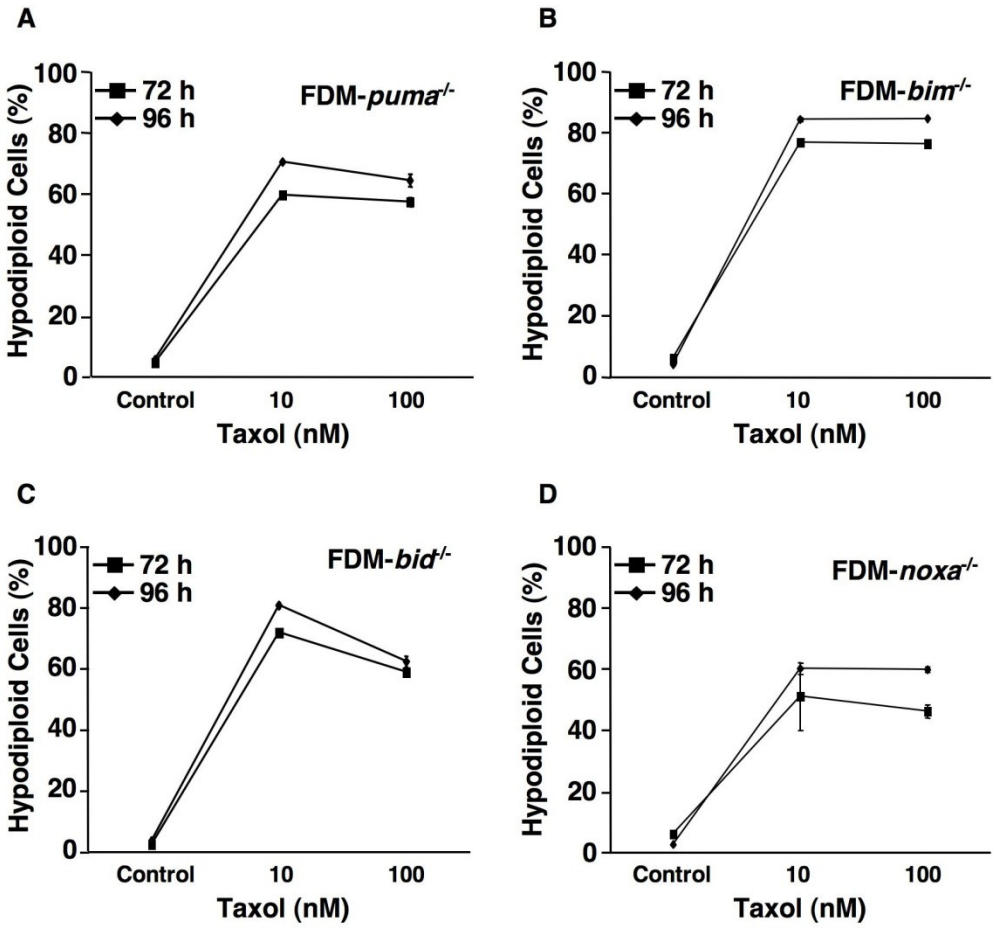

Figure 25: Taxol induces apoptosis but fails to induce polyploidy in *bid, bim, noxa, puma* deficient and wild type FDM cells. FDM-*bid*$^{-/-}$, FDM- *bim*$^{-/-}$, FDM-*noxa*$^{-/-}$, FDM-*puma*$^{-/-}$ cells were cultured with different concentrations of taxol for different durations as depicted and subjected to flow cytometric analysis of DNA content. Cells with a sub-G1 hypodiploid DNA content were considered apoptotic. (A) Taxol induced apoptosis in FDM-*puma*$^{-/-}$ cells (B) Taxol induced apoptosis in FDM- *bim*$^{-/-}$ (C) Taxol induced apoptosis in FDM-*bid*$^{-/-}$ and (D) FDM-*noxa*$^{-/-}$ cells. The results represent means ± SD of triplicates.

Treatment of cells with 100 nM taxol for 72 h was chosen to further analyze induction of polyploidy in relation to apoptosis. Cells were incubated for 72 h in the presence or absence of 100 nM taxol and induction of apoptosis and polyploidy were determined by flow cytometry. As shown in Figure 26A, FDM WT exhibited enhanced apoptosis and cells deficient for *bid, bim*, *noxa* and *puma*, respectively, did not show a reduction in apoptosis induction. Besides, these cells did not show an enhanced hyperploid DNA content (Figure 26B & C), confirming earlier data that apoptosis sensitive cells show no induction of polyploidy.

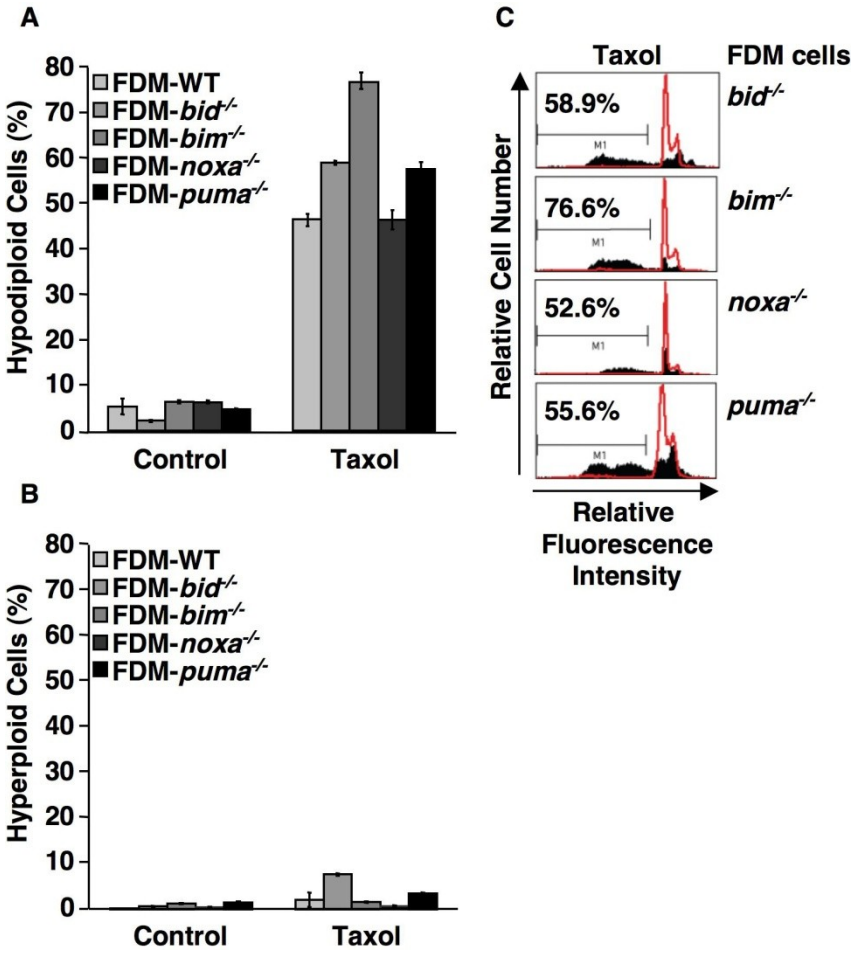

Figure 26: Taxol induces apoptosis but fails to induce polyploidy in WT, *bid, bim, noxa* and, *puma* deficient FDM cells. FDM-WT, FDM-bid$^{-/-}$, FDM- *bim*$^{-/-}$, FDM-*noxa*$^{-/-}$, FDM-*puma*$^{-/-}$, cells were cultured for 72 h in the presence or absence of 100 nM taxol and subjected to flow cytometric analysis of DNA content. (A) Cells with a sub-G1 hypodiploid DNA content were considered apoptotic. (B) Cells showing a hyperploid (>4N) DNA content were considered polyploid. (C) Representative DNA histograms for 100 nM taxol-induced genomic DNA fragmentation histograms (in black) overlayed with control histograms (red). Percentages of apoptotic cells are given in the histograms for taxol treated cells. The results represent means ± SD of triplicates.

3.13 Apoptosis induced by the microtubule inhibitors is caspase dependent and inhibition of caspase activity leads to polyploidy

To preclude the possibility of a caspase independent induction of cell death, cells were preincubated with the pan-caspase inhibitor Q-VD-OPh for 2 h prior to taxol treatment. Burkitt lymphoma cells were harvested after 72 h of taxol treatment and apoptosis was determined by flow cytometry.

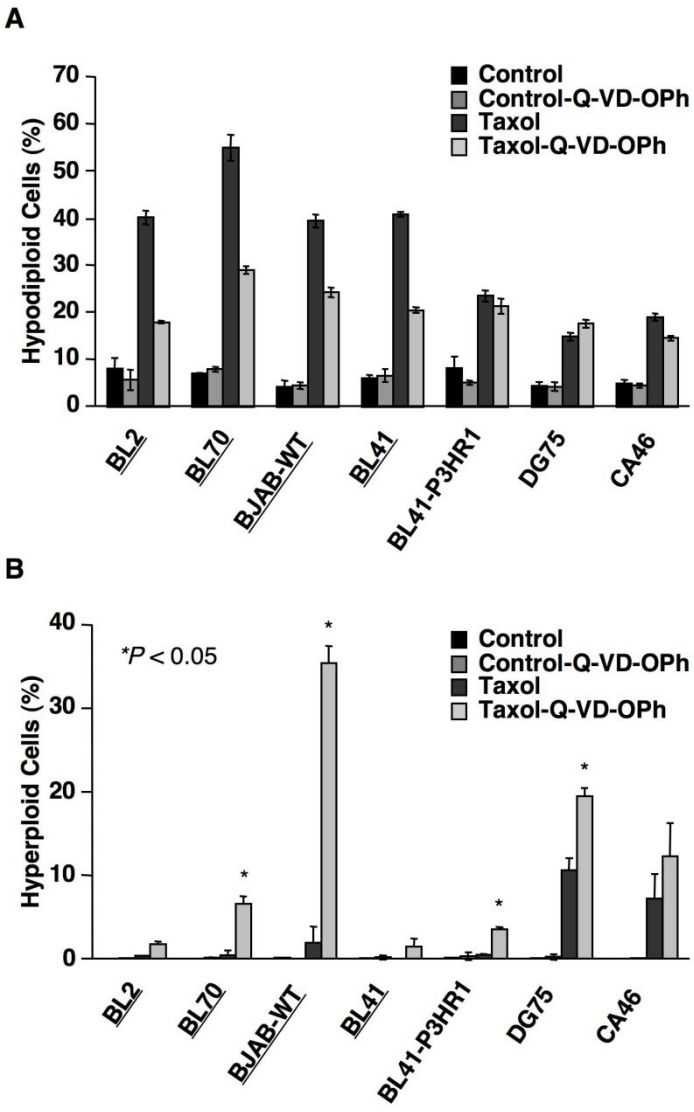

Figure 27: Inhibition of caspase activity leads to decreased induction of apoptosis and enhanced polyploidy in taxol treated Burkitt lymphomas cells. Indicated cell lines were cultured for 72 h in presence or absence of 100 nM taxol and/or 10 µM pan-caspase inhibitor Q-VD-OPh and subjected to flow cytometric analysis of DNA content. Control indicates the cells cultured in medium with out microtubule inhibitor treatment. (A) Cells with sub-G1 hypodiploid DNA content were considered apoptotic. (B) Cells showing hyperdiploid (>4N) DNA content are considered polyploidy. Asterisks indicate statistical significance calculated using Student's t test ($p < 0.05$). The results represent means ± SD of triplicates.

The pan-caspase inhibitor Q-VD-OPh inhibited apoptosis in the apoptotic sensitive cells. For example, in BJAB-WT cells apoptosis was reduced from 39% to 24%, which accounts to a 39% reduction (Figure 27A). There was little or no effect in apoptosis resistant cells. When analyzed for hyperploid DNA content, most of the cell lines sensitive to apoptosis induced by taxol, incited polyploidy. The stongest effect was seen in BJAB-WT cells where up to 35%

displayed polyploidy when apoptosis was inhibited by pan-caspase inhibitor Q-VD-OPh compared to 2% of cells with taxol treatment alone (Figure 27B). Similarly, other apoptotic sensitive cell lines also displayed enhanced polyploidy after inhibition of caspase for which significance was calculated, indicating that deregulation of caspase activation can trigger polyploidization upon culture in the presence of microtubule inhibitors.

To verify whether nocodazole treatment also incites polyploidy in the presence of a pan-caspase inhibitor as observed in taxol treated cells, cells were preincubated with the pan-caspase inhibitor Q-VD-OPh for 2 h prior to nocodazole treatment. Cells were harvested after 72 h of nocodazole treatment and apoptosis was determined using flow cytometry. The pan-caspase inhibitor Q-VD-OPh inhibited apoptosis to a large extent in apoptosis sensitive cells. For example, in BJAB-WT cells apoptosis was reduced from 36% to 20%, which accounts to a 44% reduction (Figure 28A). Caspase inhibition had little effect on apoptosis resistant cells similar to the observations made with taxol treatment. Whereas, inhibition of nocodazole-induced apoptosis by pan-caspase inhibitor triggered cells to undergo polyploidy. The strongest effect was seen again in BJAB-WT cells where 45% showed polyploidization when treated with nocodazole and pan-caspase inhibitor Q-VD-OPh as compared to 2% in cells with nocodazole treatment alone (Figure 28B). This clearly indicates that taxol and nocodazole induce apoptosis in a caspase dependent manner and inhibition of caspase activity leads to polyploidization. This implies that apoptosis might serve as a fail-safe mechanism to eliminate cells with disturbed ploidy.

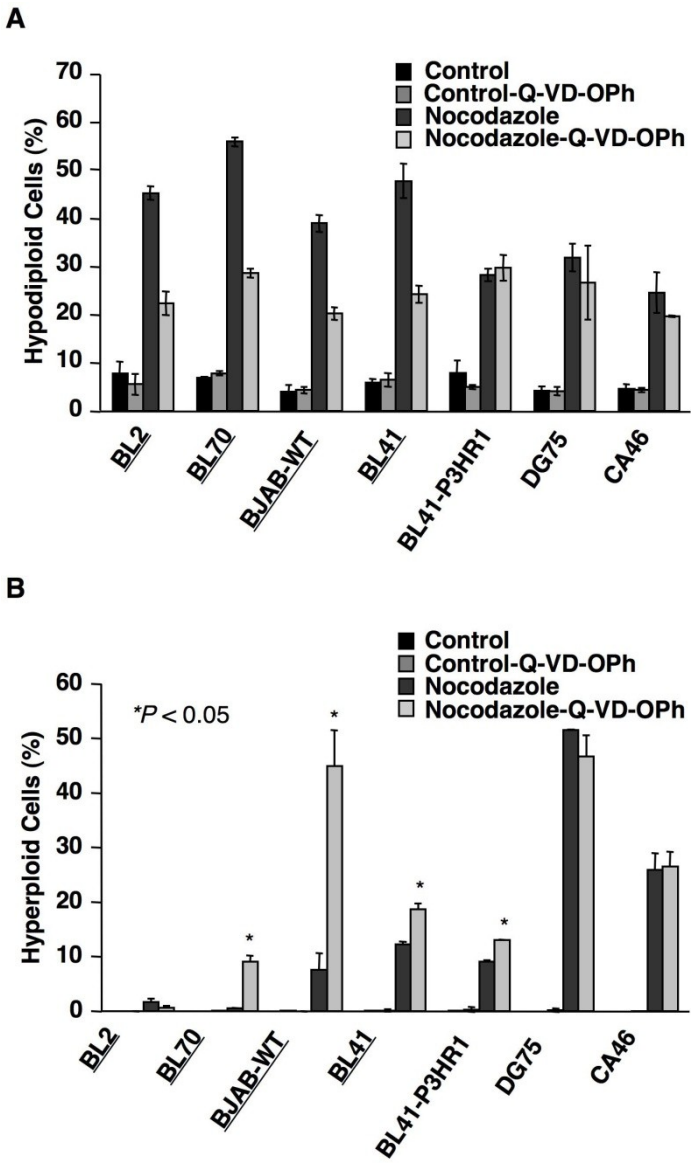

Figure 28: Inhibition of caspase activity leads to decreased induction of apoptosis and promotes polyploidy in nocodazole treated Burkitt lymphoma cells. Indicated cell lines were cultured for 72 h in presence or absence of 100 nM nocodazole and/or 10 μM pan-caspase inhibitor Q-VD-OPh and subjected to flow cytometric analysis of DNA content. Control indicates the cells cultured in medium with out microtubule inhibitor treatment. (A) Cells with sub-G1 hypodiploid DNA content were considered apoptotic. (B) Cells showing hyperdiploid (>4N) DNA content are considered polyploid. Asterisks indicate statistical significance calculated using Student's t test ($p < 0.05$). The results represent means ± SD of triplicates.

It is interesting to know what happens in cells treated with vincristine and a pan-caspase inhibitor, as vincristine did not promote polyploidy in cells resistant to apoptosis as shown in Figure 11A-B. Cells were treated similarly with the pan-caspase inhibitor Q-VD-OPh and vincristine and subjected to flow cytometric analysis of apoptosis and polyploidy. A

decrease in apoptosis induced by vincristine treatment was observed, when cells were additionally incubated with the pan-caspase inhibitor Q-VD-OPh. For example, in BJAB-WT cells apoptosis was reduced form 51% to 30%, which accounts to a 42% reduction (Figure 29A), similar to the observations made with taxol and nocodazole treatment. However, less than 3% cells showed polyploidy induction upon caspase inhibition and vincristine treatment (Figure 29B), which is in contrast to taxol and nocodazole treatment. This further confirms earlier results, that vincristine fails to induce polyploidy.

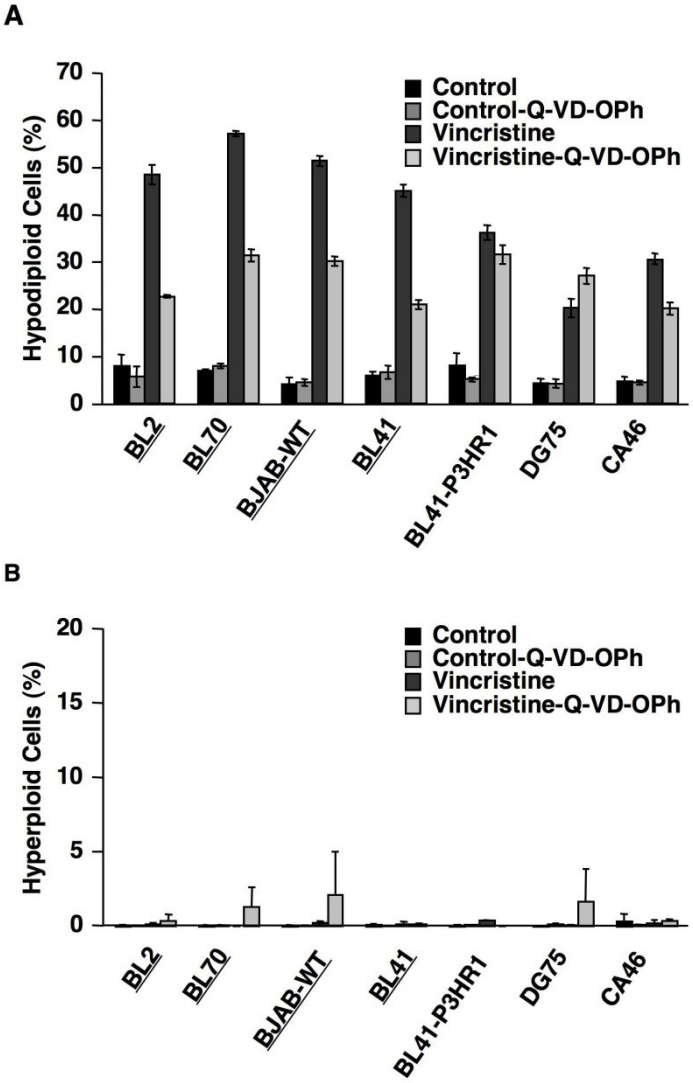

Figure 29: Inhibition of caspase activity leads to decreased induction of apoptosis and does not induce polyploidy in vincristine treated Burkitt lymphomas. Indicated cell lines were cultured for 72 h in presence or absence of 100 nM vincristine and/or 10 µM pan-caspase inhibitor Q-VD-OPh and subjected to flow cytometric analysis of DNA content. Control indicates the cells cultured in medium with out microtubule inhibitor treatment. (A) Cells with sub-G1 hypodiploid DNA content were considered apoptotic. (B) Cells showing hyperdiploid (>4N) DNA content are considered polyploid. The results represent means ± SD of triplicates.

3.14 Caspase-8 plays an important role in taxol induced apoptosis

To test if caspase-8 inhibition abrogates taxol induced cell death, cells were incubated for 2 h with caspase-8 fmk inhibitor z-IETD-fmk prior to taxol treatment. Cells were harvested after 72 h and analyzed for apoptosis. Taxol sensitive cells showed reduced apoptosis as compared to the control untreated cells, when analyzed for hypodiploid DNA using modified a cell cycle assay. For example, in BJAB-WT cells apoptosis was reduced from 46% to 24%, which accounts to a 48% reduction (Figure 30). This supports the possibility of caspase-8 activity being involved in Bid cleavage (Figure 17) thereby, contributing to cellular execution.

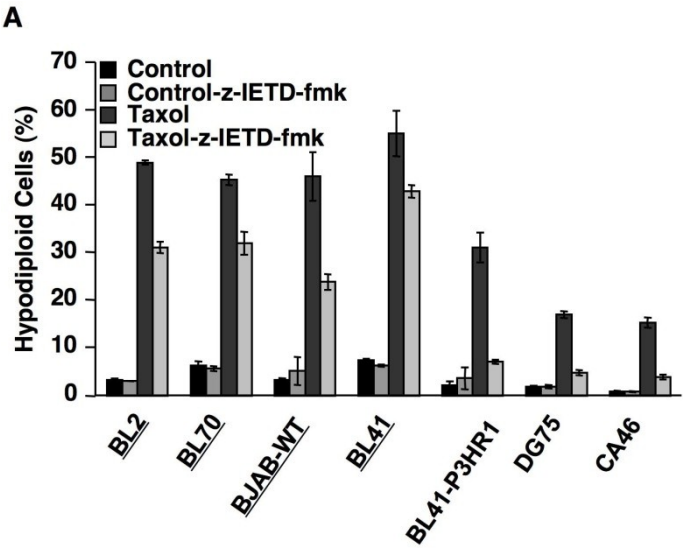

Figure 30: Caspase-8 inhibition represses apoptosis induced by taxol in Burkitt lymphoma cells. Indicated cell lines were cultured for 72 h in presence or absence of 100 nM taxol and/or 20 µM caspase-8 inhibitor and subjected to flow cytometric analysis of DNA content. Control indicates the cells cultured in medium with out microtubule inhibitor treatment. Cells with sub-G1 hypodiploid DNA content were considered apoptotic. The results represent means ± SD of triplicates.

Understanding the relation between cell cycle deregulation and induction of apoptosis by microtubule inhibitors is crucial for improving therapy and consequently improving the prognosis of cancer patients. Polo like kinase which is involved in G2/M cell cycle checkpoint regulation and known to be upregulated in most of the cancers, was used to deregulate mitotic cell cycle checkpoint control by ectopic over-expression of PLK1 (Polo like kinase 1), PBD (Polo box domain of PLK1) and KD (Kinase domain of PLK1). The aim was to investigate if manupulation of PLK1 has any added benefit to chemotherapy in aggressive and resistant cancers.

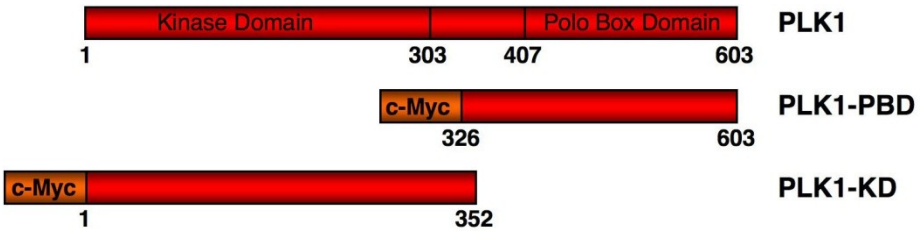

Figure 31: Illustration depicts the structure of PLK1 and its mutants cloned for the intended study

In this approach, the open reading frames of PLK1, PBD and KD were cloned into inducible mammalian expression plasmid pRTS-1. pRTS-1 is a novel, EBV-derived episomally replicating plasmid that carries all the elements for conditional expression of a gene of interest via Tet regulation (Georg W. Bornkamm et al.). The vector is characterized by low background activity and high inducibility in the presence of tetracycline (tet) or doxycycline (Dox). The gene of interest is expressed from the bidirectional promoter P_{tet}bi-1 that allows simultaneous expression of two genes, of which one may be used as surrogate marker like GFP for the analysis of expression of the gene of interest. Tight downregulation is achieved through binding of the silencer tTS^{KRAB} to P_{tet}bi-1 in the absence of Dox. Addition of Dox releases repression and, via binding of $rtTA2^{S}$-M2, activates P_{tet}bi-1. Similar cloning efforts were made before hand in pcDNA3 and an adeno associated viral (AAV) construct. Due to the low and, incase of AVV, cell line variable low transfection and transduction efficiency, these experiments were abandoned in favour of pRTS-1.

3.15 Induction of apoptosis upon deregulation of PLK1 function in Burkitt lymphomas

CA46, BL41, BJAB cells were transfected with PLK1, PBD and KD, respectively by electroporation and the transfectants were selected in presence of hygromycin concentration respectively for 4 weeks. These cells were checked for overexpression of PLK1 using an anti-PLK1 antibody and PBD, KD with c-Myc antibody by immunoblotting. Figure 32A depicts the overexpression of PLK1, c-Myc tagged PBD and c-Myc tagged KD in CA46 Burkitt lymphoma cells after incubating cells in presence of doxycycline for 24 h, 48 h and 72 h. Only the respective transfectants showed overexpression but not the mock transfected cells confirming the functionality of the cell lines.

To assess if deregulation of PLK1 function would lead to induction of apoptosis, BL41 transfectants were induced to overexpress PLK1, PBD, KD for different time points as indicated in Figure 32B. Apoptosis was determined by flow cytometry using a modified cell cycle assay for measurement of hypodiploid DNA content in the GFP positive cells, which were also considered positive for the gene expression. Only GFP positive cells were gated due to lack of 100% positive transfectants, which can be ascribed to few untransfected cells resistant to hygromycin selection and their overgrowth with time compared to positive transfectants. PLK1, PBD or KD overexpression did not induce enhanced apoptosis in BL41 cells when compared to mock transfectants, Of note, there was slight induction of apoptosis up to 10% at 48 h time point in BL41 cell line upon PBD overexpression compared to <2% of control cells.

To verify, if the observations made in BL41 cells overexpressing PLK1, PBD, KD or mock are ubiquitous among Burkitt lymphoma cells, CA46 and BJAB transfectants were induced to express PLK1 or the mutants and were analyzed for the apoptosis at the indicated time points as shown in Figure 32C-D. Apoptosis was determined by flow cytometry using a modified cell cycle assay for measurement of hypodiploid DNA content in GFP positive cell population. Similar to earlier observation in BL41 cells, CA46 and BJAB transfectants did not induce high levels of apoptosis upon overexpression of PLK1, KD, and PBD. This implies that PLK1 deregulation alone is not sufficient to induce enhanced apoptosis in Burkitt lymphomas.

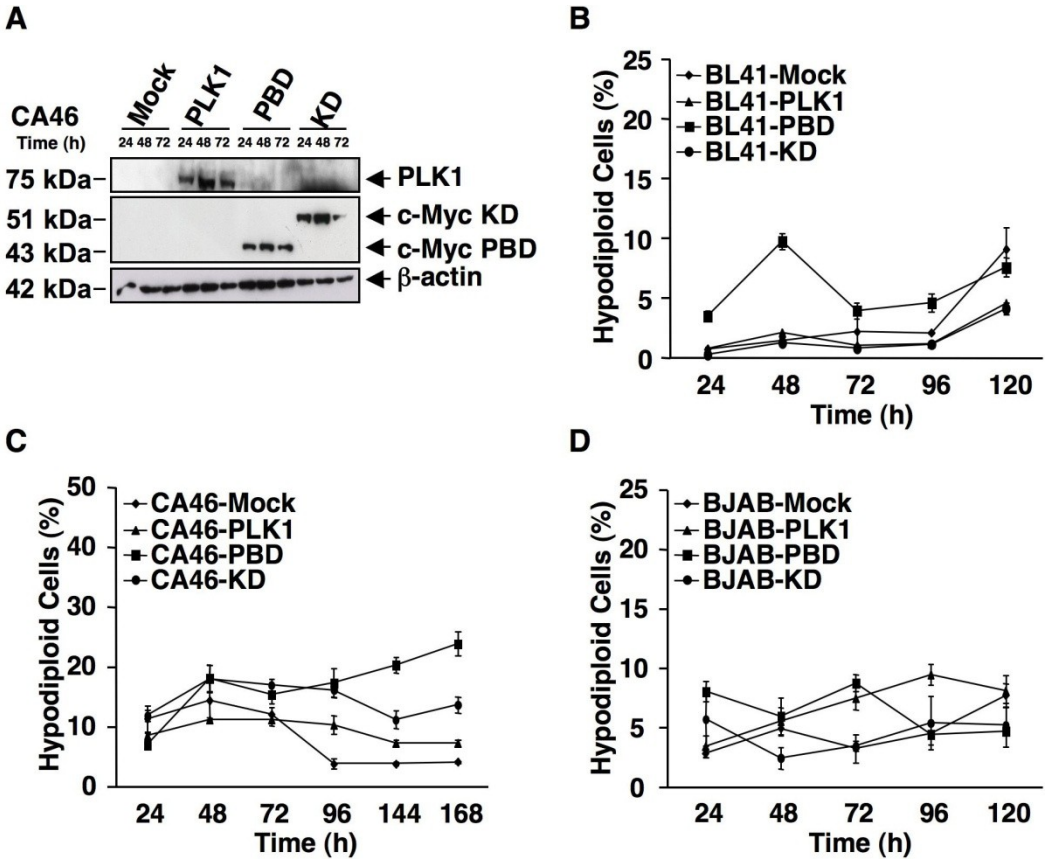

Figure 32: Ectopic overexpression of PLK1, PBD and KD respectively failed to induce high rates of apoptosis in BL41, BJAB and CA46 cells. BL41, BJAB and CA46 cells were transfected with pRTS-1 plasmid carrying PLK1, PBD or KD respectively and empty vector as control. Cells were cultured for indicated time points in the presence of doxycycline (Dox) to induce expression. (A) Immunoblot analysis of PLK1, PBD and KD expression levels in different CA46 cells. GFP positive cells were gated which are also considered positive for PLK1, PBD or KD expression and the gated population were analyzed for DNA fragmentation for indicated time points (B) in CA46 cells (C) in BL41 cells and (D) in BJAB cells. Cells with sub-G1 hypodiploid DNA content were considered apoptotic. Data shown are means ± SD of triplicates.

3.16 PLK1 deregulation leads to G2/M cell cycle arrest, while PLK1 overexpression promotes cell cycle progression

Combinatorial drug therapy is a treatment paradigm that has proved to be effective in cancer. Towards this approach and to determine the function of PLK1 in taxol-induced apoptosis and ploidy control, the effect of PLK1, KD or PBD and in combination with taxol on cell cycle distribution was analyzed. The respective BL41 transfectants were cultured in the presence of doxycycline. After 24 h of induction, GFP positive cells were sorted by FACS.

These cells were then treated for 24 h and 48 h with taxol and were assayed for cell cycle distribution. Time points shown in Figure 33A are total time of treatment starting from initial induction of cells with doxycycline. As shown in Figure 33A, overexpression of PLK1 showed a slight increase in the G2/M cell population to 19.0% and 25.2% compared to 6.5% and 19.8% in mock cells by 48 h and 72 h, respectively. It was shown Figure 11 and Figure 33 that taxol induces accumulation of cells in the G2/M phase. Overexpression of PLK1 in addition to taxol treatment showed, however, a reduction of G2/M phase arrested cells accounting to 35.2% by 48 h and 64.4% by 72 h compared to 63.0% and 81.1% in taxol treated mock cells.

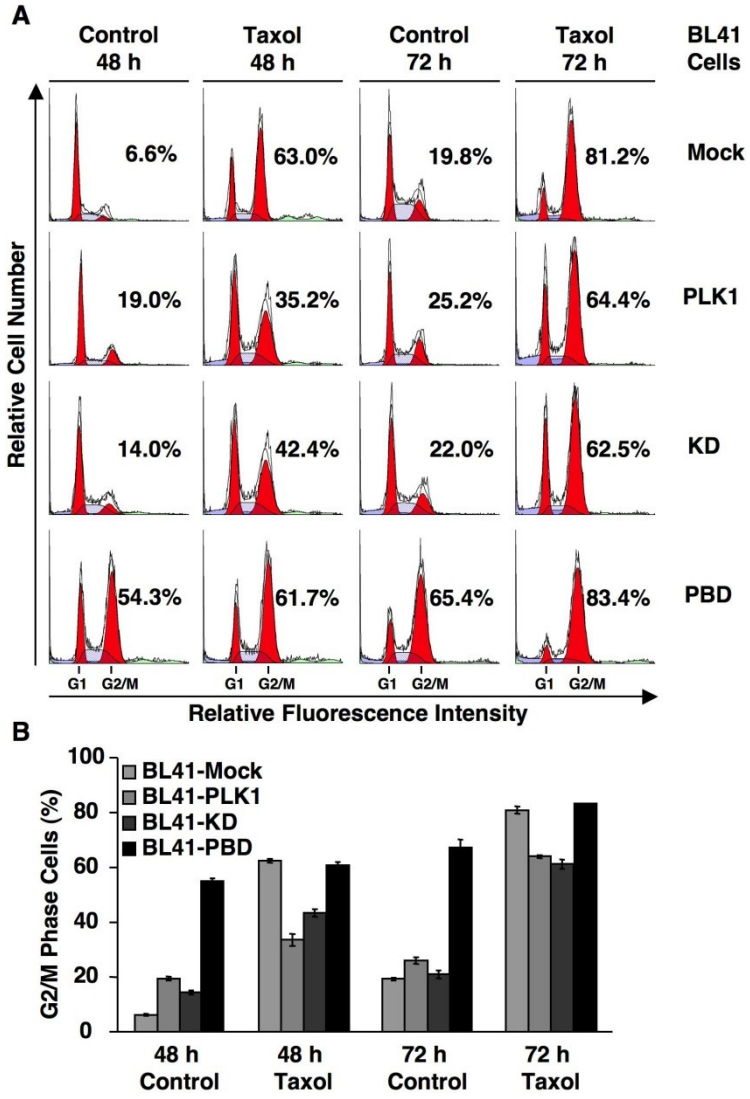

Figure 33: Overexpression of PLK1 leads to reduction in taxol-mediated G2/M arrested cell population. BL41 cells transfected with mock, PLK1, PBD or KD were induced with doxycyclin for 24 h and GFP positive cells were FACS sorted. These sorted cells were cultured for 48 h and 72 h in presence or absence of 100 nM taxol and subjected to flow-cytometric analysis of cell cycle. (A) Linear emission of PI dye from cells was measured and percentage G2/M (4N) DNA content was analyzed using MODFIT-LT software. Left red peak = G0/G1; right red

peak = G2/M; hatched peak = S; (B) representative bar graph of the percentage G2/M cells. Data shown are means ± SD of triplicates.

Supporting the PLK1 effect on BL41 cells, overexpression of KD also diplayed a slightly higher G2/M population of 14.0% and 22.0% compared to mock cells displaying 6.5% and 19.8% by 48 h and 72 h respectively. Additional treatment with taxol showed a reduced percentage of G2/M phase arrested cells in the KD transfectants accounting to 42.4% by 48 h and 62.5% by 72 h compared to 63.0% and 81.2% in taxol treated mock cells. This clearly suggests that excessive kinase activity of PLK1 assists cell cycle progression, ascribing why PLK1 is highly expressed in most of the cancer cells.

In PBD overexpressing cells, G2/M arrest is more pronounced displaying 54.3% and 65.4% G2/M cell cycle arrested cells compared to control transfectants with 6.6% and 19.8% by 48 h and 72 h, respectively (Figure 33A-B). Additional treatment with taxol had only a slight effect by enhancing G2/M arrested cell population to 61.7% and 83.4% by 48 h and 72 h, respectively which were similar to the percentages of G2/M arrested cell population exhibited by taxol treated mock cells. Of note, abrogation of PLK1 function hinders the cell cycle progression by activating G2/M checkpoint control and, in combination with taxol, has little effect on PBD induced G2/M cell cycle arrest.

3.17 PLK1 deregulation triggers G2 and mitotic arrest

To further analyze mitotic entry in BL41 cells overexpressing PLK1, PBD or KD with regard to taxol treatment, BL41 cell transfected with PLK1, PBD, KD or mock were cultured in the presence of doxycycline to induce transgene expression. After 24 h of induction, GFP positive cells were sorted by FACS. These cells were additionally treated for 24 h and 48 h with taxol and examined by mitosis-specific MPM2 staining. The MPM2 antibody detects mitosis specific phospho-epitopes, e.g. in topoisomerase-IIα. The MPM2 positive cells were then analyzed by flow cytometry. After 48 h of PLK1 and KD overexpression, 7.2% and 5.5% cells, respectively were observed to be in the mitotic phase of cell cycle which was comparable to mock cells with 2.2% mitotic cell population (Figure 34A and left column of Figure 34B) and this proportion remained more or less the same by 72 h (Figure 34A). Additional treatment with taxol greatly enhanced mitotic arrest in mock transfectants displaying 40.1% mitotic cells by 48 h. But, PLK1 and KD overexpression together with taxol treatment induced mitotic arrest to a much lesser extent, exhibiting 19.5% and 20.8% mitotic cells, respectively indicating release from mitotic arrest (Figure 34A and right column

of Figure 34B). This proportion remained similar for 72 h (Figure 34A). These results strengthened the earlier data indicating that the kinase activity of PLK1 promotes cell cycle progression and mitotic exit.

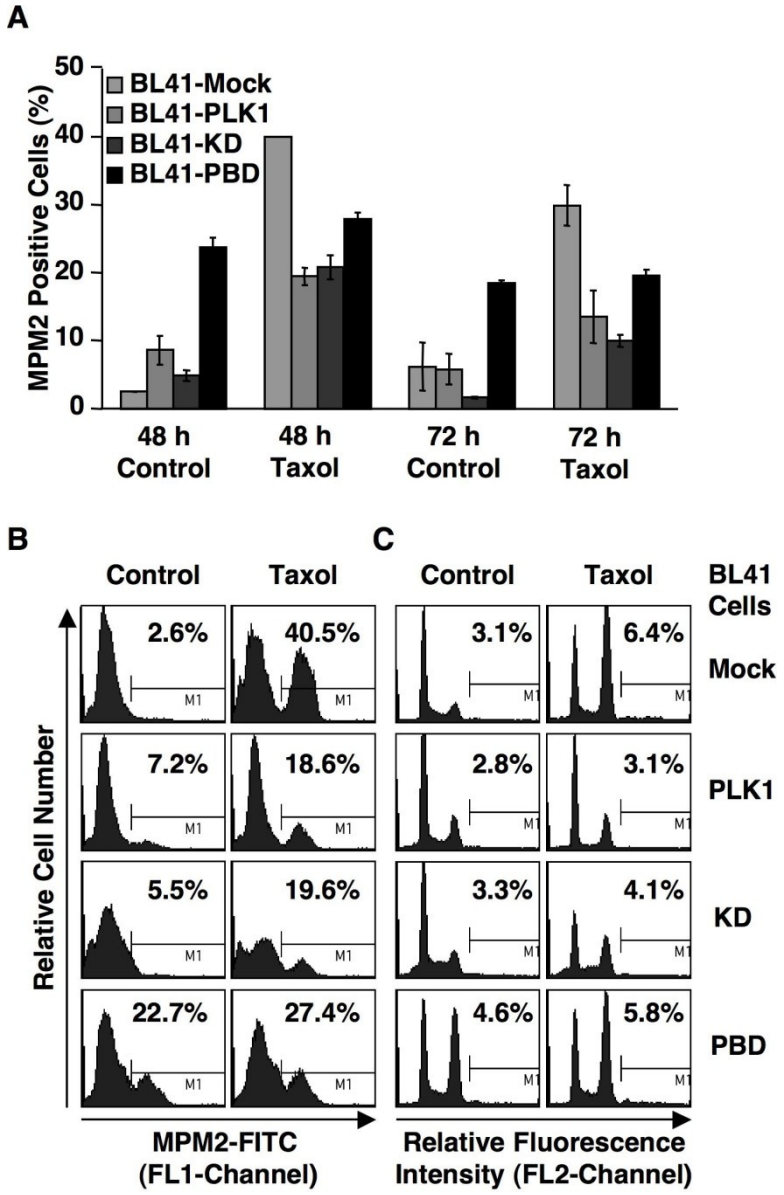

Figure 34: Ectopic overexpression of PLK1, PBD or KD in BL41 cells in combination with taxol shows a differential impact on mitotic cells. BL41-mock, -PLK1, -PBD or -KD cells were induced with doxycyclin for 24 h and GFP positive cells were FACS sorted. These sorted cells were cultured for 48 h and 72 h in the presence or absence of 100 nM taxol. Cells were stained with a fluorescein isothiocyanate (*FITC*)-labeled MPM2 antibody. Bi-variate analysis of DNA content (propidium iodide) and MPM2 antibody staining were performed, and 4N, MPM2-positive, *i.e.* M phase cells were identified by gating. (A) Data are given as percentage of mitotic cells from total cell population. (B) Representative histograms for 48 h showing percentages of 4N cells positive for

MPM2 indicated between markers. (C) Representative histograms for 48 h showing percentage of cells with hyperdiploid (>4N) DNA content which were considered polyploid. Data shown are means ± SD of triplicates.

In contrast to PLK1 and KD, cells overexpressing PBD displayed an increased percentage of mitotic cells accounting up to 23.7% compared to 2.6% in mock transfectants. Additional treatment with taxol for 48 h, manifested limited accumulation of the mitotic cell population accounting 27.9% in PBD overexpressing cells compared to 40.1% in mock cells. This proportion remained similar for 72 h (Figure 34A). From earlier data (Figure 33A-B) it is known that PBD overexpression in combination with taxol and taxol treated mock cells showed similar percentage of a G2/M population. Interestingly, this does not appear to be the same case with mitotic index. This indicates that overexpression of PBD decreases the mitotically arrested cell population upon taxol exposure, possibly due to enhanced check point activation at the G2 phase of the cell cycle.

To verify whether deregulation of PLK1 function would lead to polyploidy, cells overexpressing PLK1, PBD or KD were further analyed for hyperdiploid DNA content in the presence or absence of taxol. There was no enhanced polyploidy in any of the transfectants compared to mock cells (Figure 34C), implying that deregulating PLK1 function does not induce polyploidy in BL41 cells regardless of taxol treatment.

3.18 Similar cell cycle characteristics observed in different Burkitt lymphomas: DG75 and CA46 in response to PBD overexpression.

Furthermore, to understand if the observed effect of G2 and mitotic arrest in BL41 cells is a general phenomenon among resistant Burkitt lymhomas in response to PLK1 deregulation, DG75 and CA46 transfectants were analyzed as shown in Figure 35. After 72 h of induction of gene expression, apoptosis was determined by flow cytometric measurement of hypodiploid DNA content. Similar to earlier observations, PBD overexpression in BL41 cells led to a slight induction of apoptosis. DG75 and CA46 cells also showed apoptosis induction upon PBD expression, albeit to a lesser extent as compared to BL41 cells (Figure 35A-B). DG75 and CA46 cells overexpressing PBD displayed, however, increased G2/M arrest up to 72% and 68%, respectively, with mock cells showing less than 15% 4N cells (Figure 35E-F). In concordance with BL41 cells, only a part of DG75 and CA46 cells in G2/M displayed as well markers i.e. MPM2 positivity up to 25% and 16% as compared to 8% and 7% in mock transfectants, respectively (Figure 35E-F). Yet, DG75 and CA46 cell lines are different from

BL41 in their endogeneous Bax and Bak expression. They lack Bax and showed weak Bak expression contrary to Bax and Bak proficiency in the BL41 cell line (Figure 20). All the three cell lines carry, however, p53 mutations (Figure 19).

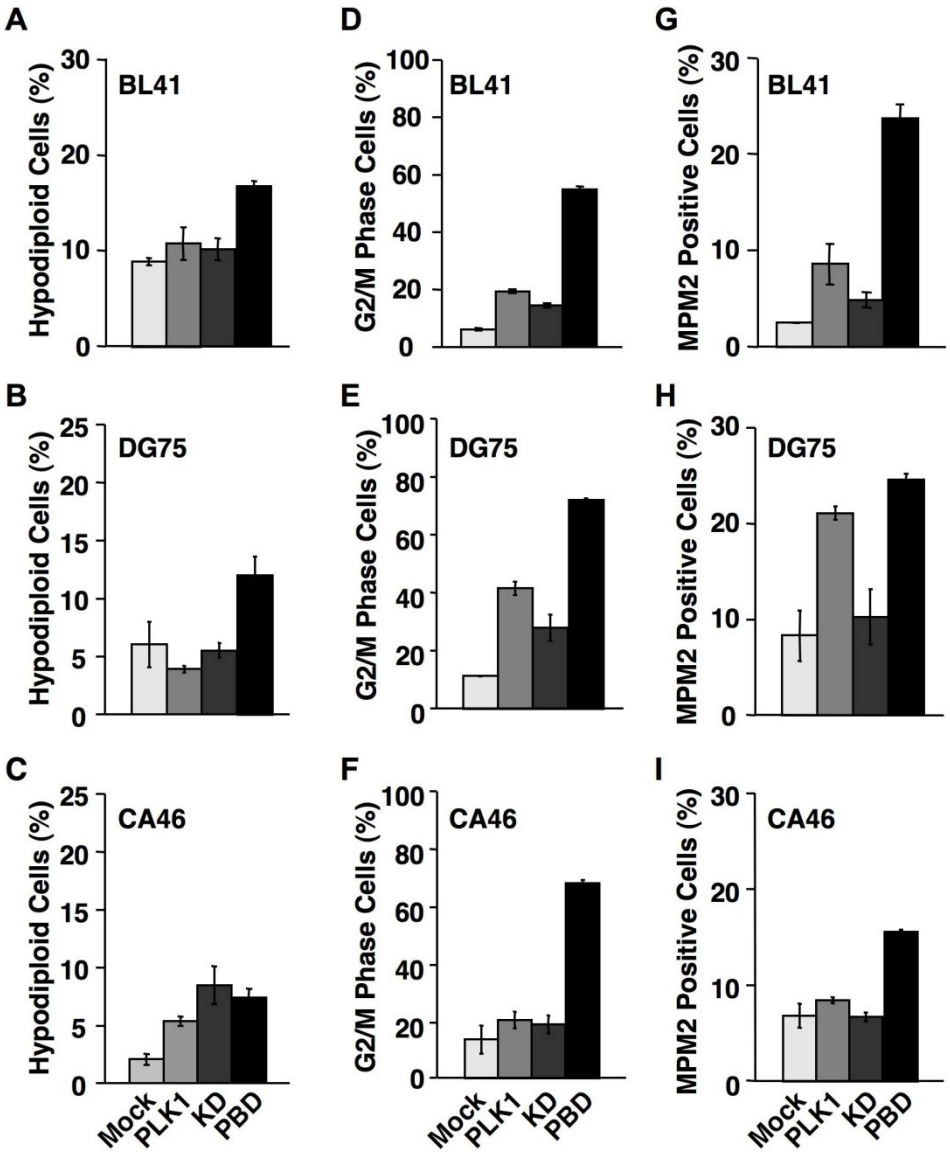

Figure 35: Deregulation of PLK1 function in apoptosis resistant DG75 and CA46 as compared to apoptosis sensitive BL41 cell line induces G2 and mitotic arrest but fails to induce prominent apoptosis. DG75 and CA46 cells were transfected with pRTS-1 plasmids carrying PLK1, PBD or KD, respectively, or the empty vector as a control. Cells were cultured for 72 h in the presence of doxycycline (Dox). (A-C) Cells were analyzed for DNA fragmentation by flow cytometry. Cells with sub-G1 hypodiploid DNA content were considered apoptotic. (D-F) Percentage of cells with a G2/M (4N) DNA content was analyzed using MODFIT-LT software. (G-I) Bi-variate analysis of DNA content (propidium iodide staining) and MPM2 antibody staining were performed, and MPM2-

positive, 4N, *i.e.* M phase cells were identified. Data are given as percentage of mitotic cells from total cell population. Data shown are means ± SD of triplicates.

3.19 PLK1 deregulation promotes polyploidy in Burkitt lymphomas lacking Bak/Bak expression

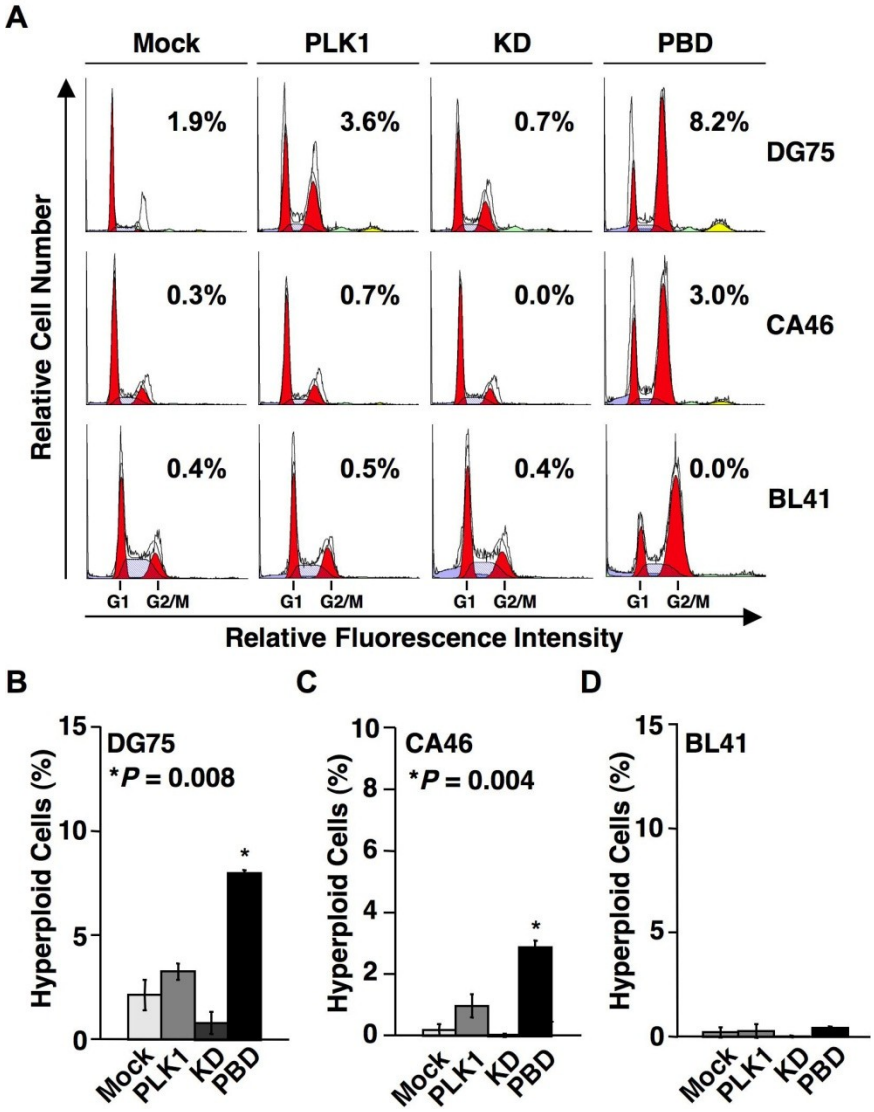

Figure 36: Burkitt lymphoma cell lines DG75 and CA46 promote polyploidy but not BL41 in response to PLK1-PBD overexpression. Data shown are from flow-cytometric analysis of cell cycle of the cells cultured for 72 h in the presence or absence of doxycyclin. (A) percentage hyperploidy (>4N DNA content) was analyzed using MODFIT-LT software. Left red peak = G0/G1; right red peak = G2/M; hatched peak = S; Yellow peak = >4N DNA content. (B) representative bar graph of the percentage of hyperploidy. Data shown are means ± SD of triplicates.

To investigate whether PBD overexpression leads to polyploidy, cell cycle distribution was analyzed in DG75, CA46 and BL41 cell lines after 72 h of induction with doxycyclin. Only DG75 and CA46 cells displayed enhanced constitutive polyploidy whose significance was measured by t-test. Upon PBD overexpression, DG75 and CA46 cells showed 8% and 3% compared to mock cells displaying 2% and 0% polyploidy cells, respectively (Figure 36B-C). Conversely, BL41 cells did not show enhanced polyploidy compared to mock cells (Figure 36D). This difference in response may be attributed to Bax and Bak proficiency that would lead to deletion of aberrant cell populations.

3.20 Deregulation of PLK1 function has an antiproliferative effect followed by necrosis in Burkitt lymphomas

Further, CA46 and BL41 transfectants were examined for their survival, as they did not undergo prominent apoptosis. As shown in Figure 37A-B, both BL41 and CA46 cell lines displayed reduced proliferation ability in response to PBD overexpression. BL41 and CA46 transfectants for PBD 72 h after induction of gene expression showed up to 50% and 38% reduction in proliferative ability (viability) respectively, when measured by XTT assay. However, there was no decrease in proliferative ability of KD and PLK1 tranfectants upon induction of gene expression, which is comparable to control cells. Further, respective transfectants were analyzed for cell death by a flow cytometric single cell propidium iodide (PI) uptake assay. Uninduced transfectants stained for PI in less than 8% of cells. When analyzed for PI uptake in the induced condition, mock and PLK1 overexpressing cells showed a slight increase in their PI uptake, which can be attributed to the background effect of the plasmid.

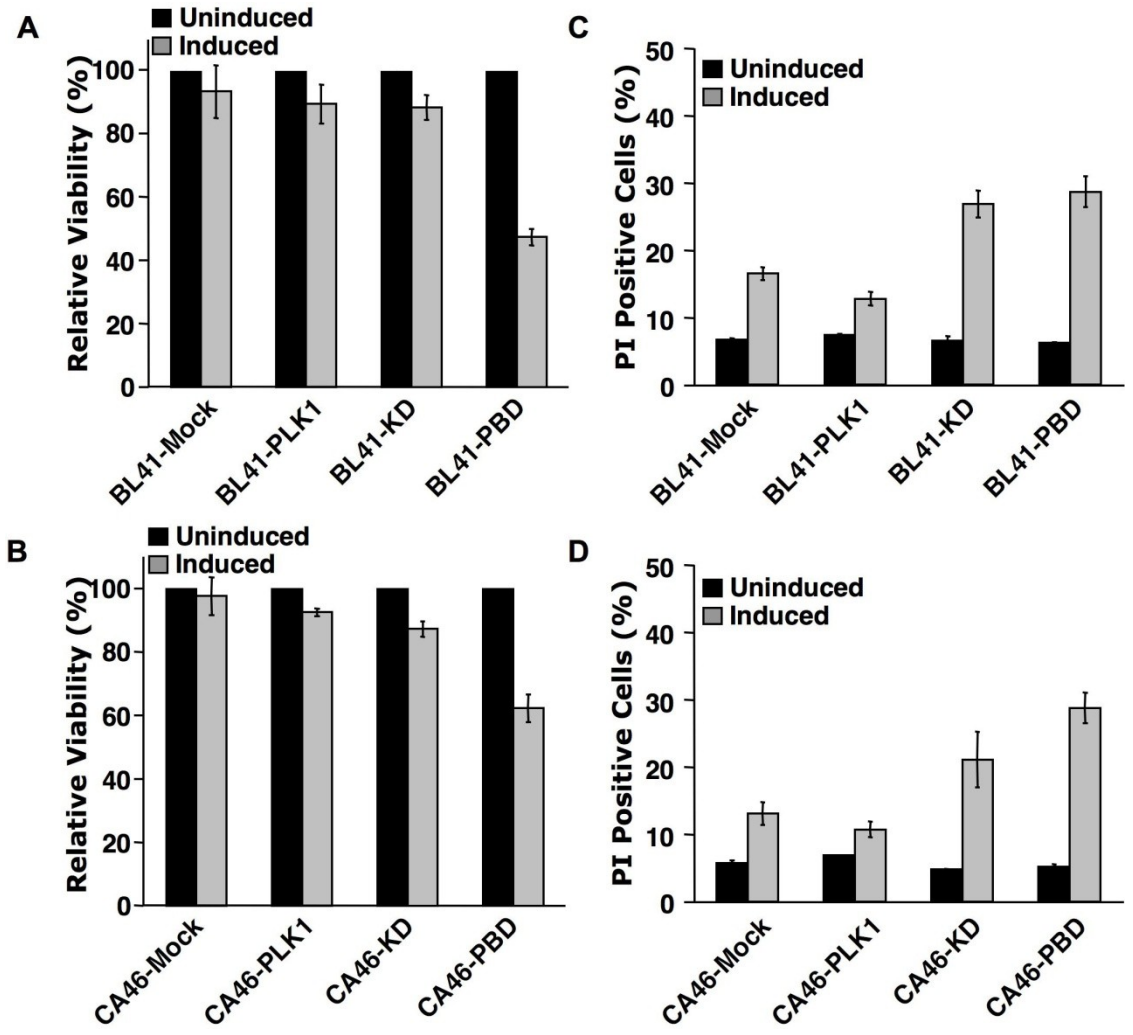

Figure 37: Abrogation of PLK1 function leads to loss of proliferative ability and induction of cell death. Cells transfected with empty vector, PLK1, PBD or KD were cultured for 72 h in the presence or absence of doxycyclin (A-B) Cell viability measurement by colorimetric XTT assay. (C-D) Cell death measurement by PI uptake. Cells were stained with PI and % PI positive (dead) cells were measured among GFP positive cells using flow cytometric analysis. Data shown are means ± SD of triplicates.

Nevertheless, PBD and KD under induced condition in BL41 cell line showed enhanced PI uptake up to 29% and 27% cells with PI positivity, respectively compared to uninduced tranfectants exhibiting below 7% cells with PI positivity (Figure 37C). Similarily CA46 transfectants of PBD and KD, under induced condition showed enhanced PI uptake up to 29% and 21% cells with PI positivity, respectively, with uninduced cells remaining below 6% (Figure 37D). These results imply that deregulation of PLK1 function leads to cell death probably in part via a necrosis like pathway in Burkitt lymphomas.

3.21 Deregulation of PLK1 and treatment with microtubule inhibitors failed to show an increase or inhibition of apoptosis induction

It is interesting to check, if deregulating the cell cycle in addition to microtubule inhibitors would lead to synergistic induction of apoptosis. BL41 cells overexpressing PLK1, PBD, KD or mock were cultured in the presence of doxycycline and additionally treated with taxol or vincristine or left untreated for 72 h. Hypodiploid DNA content was measured using flow cytometry to determine induction of apoptosis. All the cell lines left untreated exhibited approximately 9-15% background apoptosis. In contrast, taxol-treated cells showed enhanced apoptosis up to 50% in all the transfectants (Figure 38), irrespective of differences in cell cycle distribution and PI uptake shown Figure 33, 34, 37.

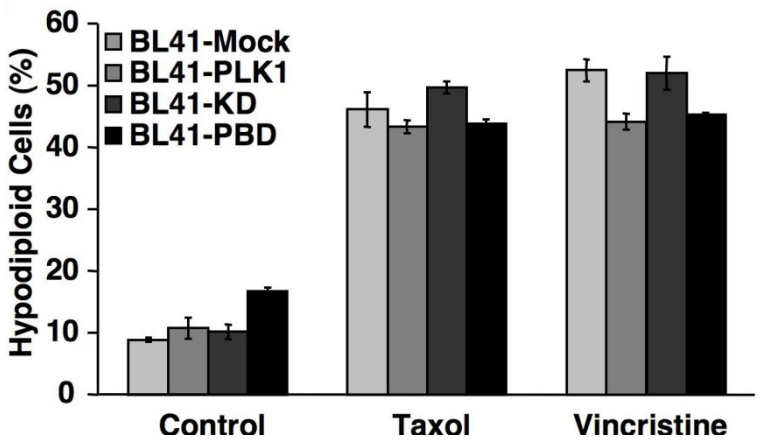

Figure 38: Microtubule inhibitor treatment and deregulation of PLK1 function show no additive or synergistic induction of apoptosis. BL41 cells were transfected with pRTS-1 plasmid carrying PLK1, PBD or KD, respectively, or empty vector as control. Cells were cultured for 72 h in the presence (on) of doxycycline (Dox) additionally in the presence or absence of 100 nM taxol or vincristine. GFP positive cells were gated which are considered positive for PLK1, PBD or KD expression and the gated populated were analyzed for DNA fragmentation by flow cytometry. Cells with sub-G1 hypodiploid DNA content were considered apoptotic. Data are means ± SD of triplicates.

Similar to observations made with taxol treatment in PLK1, PBD or KD overexpressing cells, vincristine also displayed enhanced apoptosis up to 53% in all the transfectants without any synergism, additive or inhibitory effect, disregarding cell cycle perturbation that resulted from PLK1 deregulation. This indicates that microtubule inhibitors and deregulation PLK1

function induces cell death via the same pathway. Of note, full completion of miotisis is not required for cell death induction as PBD induces M arrest.

3.22 Deregulation of PLK1 function induces apoptosis but lacks synergistic effect with microtubule inhibitors in HeLa cells

Since PLK1 deregulation studies by other groups were mainly performed in HeLa, it is interesting to see if the obsevation made with Burkitt lymphomas are similar in the HeLa cell system. In this approach, HeLa cells were transfected with PLK1, PBD and KD, respectively, and were checked for overexpression of PLK1 using PLK1 antibody by immunoblotting, while PBD and KD were detected by using c-Myc antibody. Figure 39 depicts the overexpression of PLK1, c-Myc tagged PBD and c-Myc tagged KD in HeLa transfectants after incubating cells in presence of doxycycline for 72 h. Only respective transfectants showed overexpression but not the mock transfected cells confirming the genetic status of the cell lines and transgene expression.

Overexpression of PLK1 and KD induced apoptosis up to 14% and 11% compared to <5% in control cells, indicating little or no effect of PLK1 and KD on apoptosis induction. However, deregulation of PLK1 function by overexpressing PBD induced 41% apoptosis, confirming the earlier reports that PBD overexpression induces apoptosis in HeLa cells (Fink, et al., 2007). Since deregulating cell cycle by PBD expression induced apoptosis in HeLa cells (Figure 39A), it was tempting to speculate whether PBD expression would yield a synergistic effect on apoptosis in addition to microtubule inhibitors. To test this, HeLa cell transfectants for PLK1, PBD, KD or mock were cultured in the presence of doxycycline and additionally treated with nocodazole, taxol or vincristine or left untreated for 72 h. Apoptosis and polyploidy were determined by flow cytometry using a modified cell cycle assay for measurement of hypodiploid and hyperdiploid DNA content. Additional treatment with nocodazole showed a sub-additive effect with apoptosis in around 60% in all the cells overexpressing PLK1 or PBD or KD (Figure 39B) irrespective of differences in cell cycle distribution. These results further confirm that deregulation of PLK1 function in combination with microtubule inhibitor fails to show a synergism in induction of apoptosis.

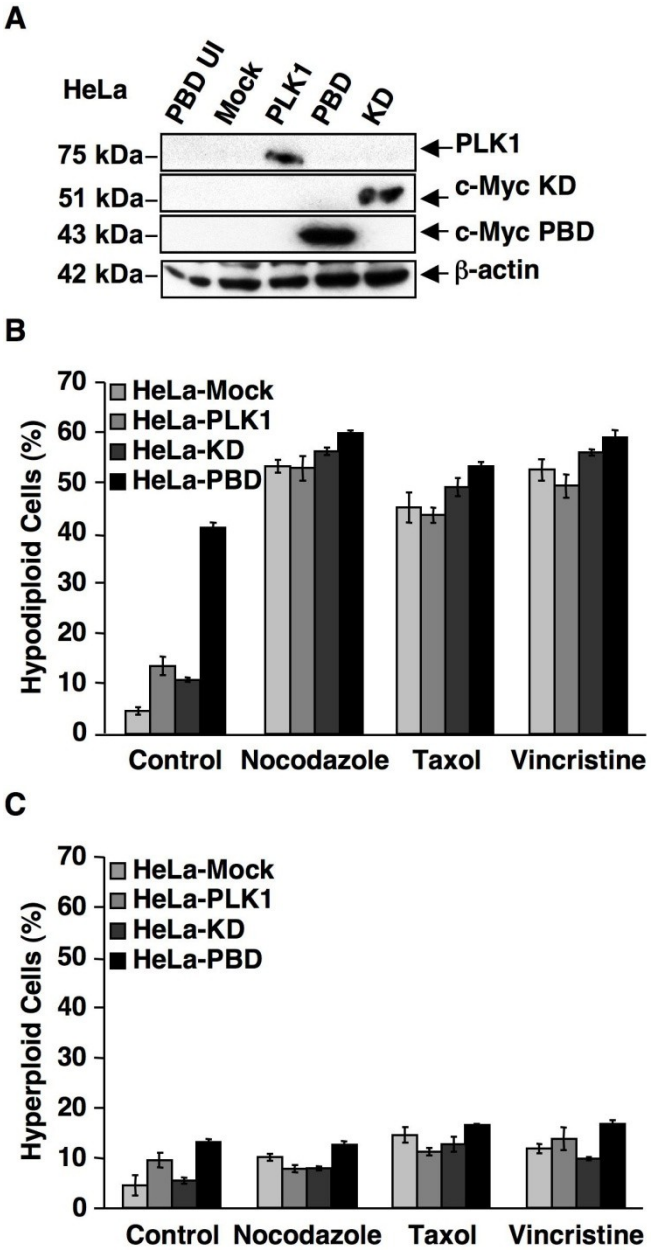

Figure 39: Polo box domain of PLK1 induces apoptosis in HeLa cells but shows no synergistic activity with microtubule inhibitors nocodazole, taxol or vincristine. HeLa transfectants of mock, PLK1, PBD or KD, respectively, were cultured for 72 h in the presence of doxycycline (Dox) (on). (A) Immunoblot analysis of PLK1, PBD and KD expression levels in HeLa cells (B) Cells were aditionally treated with 100 nM nocodazole, taxol or vincristine and analyzed for DNA fragmentation by flow cytometry. Cells with sub-G1 hypodiploid DNA content were considered apoptotic. (C) Cells showing hyperploid (>4N) DNA content were considered polyploid. Data are means ± SD of triplicates.

Concordant to observations made with nocodazole, treatment of PLK1, PBD, KD overexpressing cells with taxol, vincristine also induced apoptosis up to 53% and 59%,

respectively. This further confirms that there is no or lack of synergistic effect of PLK1, PBD and KD overexpression and microtubule inhibitor treatment (Figure 39B). At the same time when looking for polyploidy, none of the cells carrying PLK1, PBD or KD displayed enhanced enhanced number of cells with a hyperploid DNA content regardless of microtubule inhibitor treatment (Figure 39C). This supports earlier results in the Burkitt lymphoma and FDM cell systems confirming that apoptosis sensitive cell lines show no polyploidy induction.

3.23 PBD induces aberrant spindle formation and chromosomal congressional defects

To better understand the physiological importance of Plk1 localization, the phenotypes of the mitotically arrested cells produced upon PBD overexpression were studied. HeLa-PBD and HeLa-Mock (empty plasmid) stable cells were cultured in presence of doxycyclin for 48 h and were subjected to an immunofluorescence staining by using α–tubulin antibody against spindle microtubules (red) and DAPI to stain DNA (blue). The overlay of microtubule and DNA of HeLa cells with empty plasmid acting as control showed clear bipolar spindle and normal segregation of chromosomes. In response to PBD overexpression, HeLa cells in mitotic phase exhibited aberrant spindles with multipolar or bipolar microtubule arrays, but these most often had unfocussed spindle poles with lagging chromosomes (Figure 39A).

Further, similar phenotypes were observed in Burkitt lymphoma cells (BJAB cells). BJAB transient transfectants overexpressing PBD were subjected to immunofluoresence staining as stated above. Deregulation of PLK1 by PBD overexpression induced aberrant mitosis with monopolar, multipolar spindle or bipolar microtubule with lagging chromosomes. In some cases, unequal segregation of chromosomes leading to exclusion of a very small portion of DNA leading to distinct progeny with hyperploid DNA content and other one with insufficient DNA content, which would probably perish (Figure 39B). This indicates that PBD induces mitotic arrest with aberrant spindle events and chromosomal missegregation followed by chromosomal instability and cell death.

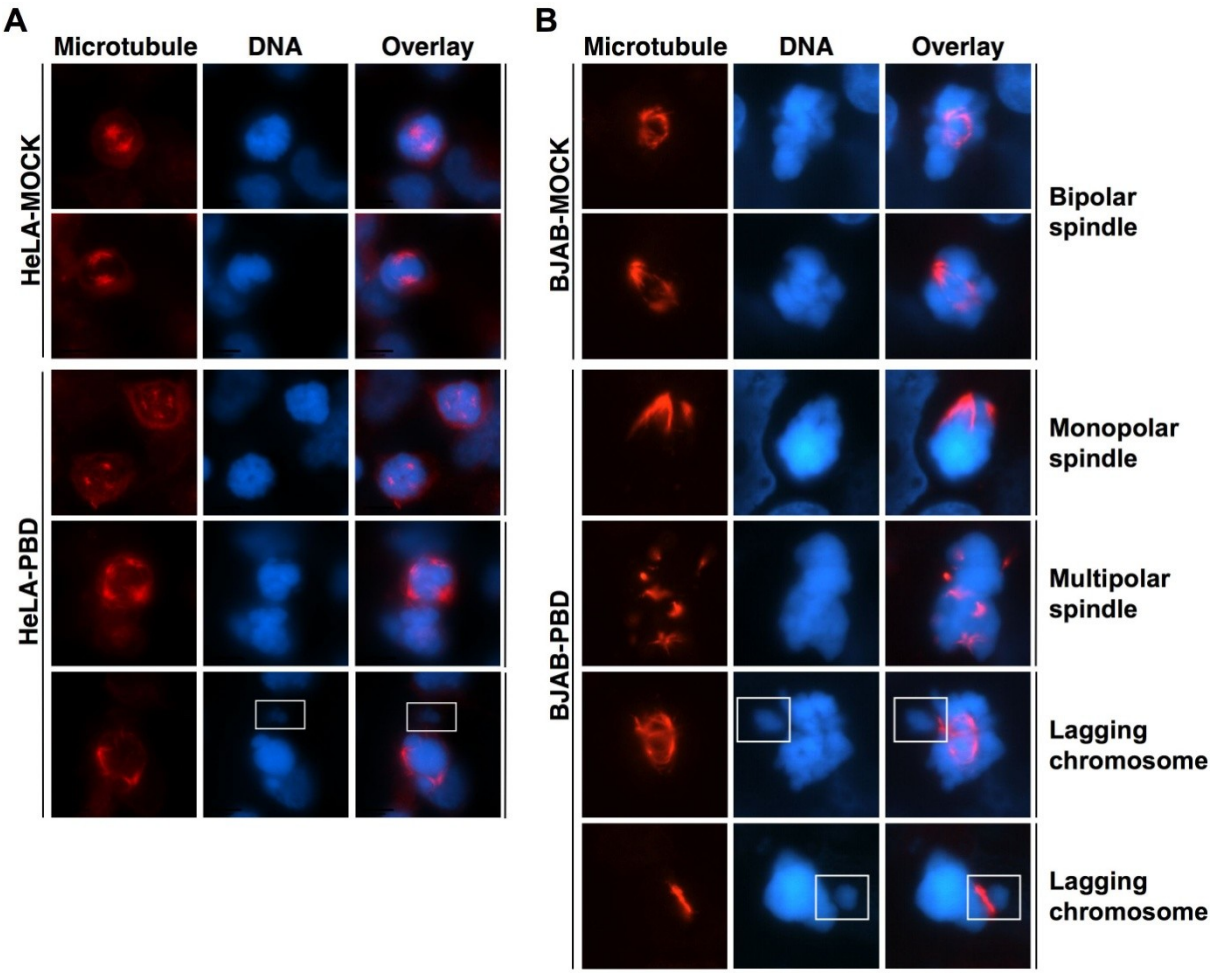

Figure 38: Deregulation of PLK1 function by PBD overexpression induces mitotic aberrations leading to chromosomal instability. The PBD stable cell line was induced for 48 h with doxycycline. After fixation and permeabilization, cells were analyzed by indirect immunofluorescence microscopy using anti--tubulin (red) antibody, and DAPI (blue) staining. (A) Mitotic phenotypes of PBD expressing HeLa cells (B) Mitotic phenotypes of PBD expressing BJAB cells.

3.24 PBD induced apoptosis in HeLa cells is caspase dependent

To investigate whether apoptosis induced by PBD overexpression is caspase dependent, cells were preincubated with pan-caspase inhibitor Q-VD-OPh for 2 h prior to doxycycline and taxol treatment, leaving unteated cells as controls. Cells were harvested after 72 h of taxol treatment and analyzed for apoptosis using flow cytometry. PBD overexpression induced apoptosis accounting up to 37% compared to 11% in uninduced cells (Figure 40A). The pan-caspase inhibitor Q-VD-OPh completely inhibited apoptosis in PBD expressing cells, down to the level of control cells (Figure 40A). Taxol alone and taxol with PBD overexpression showed apoptosis up to 53%, which was inhibited by 44% in presence of pan-caspase inhibitor Q-VD-

OPh, indicating apoptosis induced by PBD, taxol or in combination is caspase dependent in HeLa cells.

Interestingly, as shown in Figure 40B, PBD overexpressing cells cultured in the presence of the pancaspase inhibitor displayed enhanced polyploidy up to 21% by 72 h, compared to uninduced cells showing 3% polyploidy. Taxol alone upon caspase inhibition failed to induce polyploidy unlike in BL41 cell lines. Additional treatment with taxol of PBD overexpressing cells did not show synergistic polyploidy induction, which leveled out at 18%. It is clear form the data that inhibition of apoptosis induced by PBD overexpression by caspase inhibtion promotes polyploidy. This confirms the inverse relationship between induction of apoptosis and polyploidy.

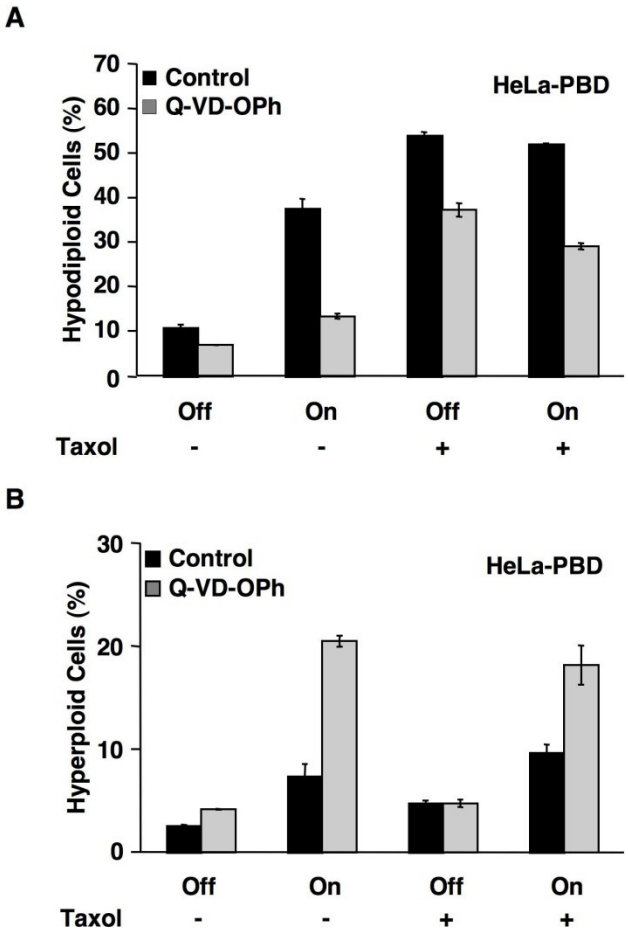

Figure 40: Inhibition of caspase activity led to inhibition of apoptosis induced by PBD and enhanced polyploidy induction in HeLa cells. HeLa-PBD cells were culture in presence (on) or absence (off) of doxycycline (Dox). (A) Cells additionally treated with or without taxol and pan-caspase inhibitor Q-VD-OPh were subjected to flow cytometric analysis of DNA content. Cells with sub-G1 hypodiploid DNA content were considered

apoptotic. (B) Cells showing hyperdiploid (>4N) DNA content are considered polyploid. Data are means ± SD of triplicates.

4 Discussion

Cancer is a genetic disease that develops through continuous accumulation of mutations and epigenetic events. Many important tumor-promoting genes have been identified during the last two decades and their functions have been well characterized (Vogelstein and Kinzler, 2004). However, recent genome-wide screenings of different tumor samples have pointed out that more gene mutations are still to be discovered and elucidation of their unique characteristics will be important to understand cancer progression (Sjoblom, 2008).

Evasion of apoptosis by tumor cells has gained much attention since it allows tumor cells to escape from elimination by endogenous default apoptosis mechanisms, immune surveillance and cancer therapeutics. Several molecular alterations that contribute to apoptosis resistance have been demonstrated but identification of novel apoptotic regulators is still important to improve tumor treatment (Malaguarnera, 2004).

Burkitt lymphomas are known as aggressive malignant B cells, as they show the highest rate of cell division among any human tumor (Hecht and Aster, 2000). Therefore, several chemotherapeutic drugs are used in cancer treatment, which generally target fast growing cells, as they are more sensitive to DNA damage and cellular stress. Polychemotherapy is also intended to circumvent rapid outgrowth of resistant clones, which represent a major therapeutic problems in Burkitt lymphomas and B-ALL therapy. Since tumor cells generally have a higher growth rate than normal cells, they are more severely affected. Development of improved therapies remains increasingly challenging, as there are certain cancer types or sub-populations, which are difficult to kill by apoptosis due to alterations in their genetic profile and as a result of acquired resistance. One category of such chemotherapeutic drugs are microtubule inhibitors, which are acclaimed as a promising therapeutic approach against a broad variety of cancer (Abal, et al., 2003; Ganansia-Leymarie, et al., 2003). The present study describes the basis of cell death mechanisms in Burkitt lymphomas upon microtubule inhibitor treatment, revealing some of the major aspects of the molecular mechanism linking apoptosis and mitotic catastrophe.

Taxol, which primarily targets the microtubule components of cytoskeleton impeding microtubule depolymerization (Wang, et al., 1999), has a broad spectrum of clinical activity against human cancers (Huizing, et al., 1995; Rowinsky, et al., 1992). In a first set of

experiments, as demonstrated in Figure 8, it was found that taxol induces apoptosis in various Burkitt lymphoma cell lines at concentrations ranging from 10 to 100 nM. This finding, together with reports from earlier publications, confirms that taxol preferentially induces apoptotic and not necrotic cell death at concentrations below 1 μg/ml in a variety of cell types, for example, B-lymphoid cells (Essmann, et al., 2000; Wieder, et al., 2001), human breast cancer cells (Charles, et al., 2001), and lung cancer cells (Das, et al., 2001). The results presented here show that taxol and nocodazole, the reversible spindle toxin that used as a control, together with vincristine induce morphological changes in Burkitt lymphoma cells leading to cell death in a time- and dose-dependent manner. Contradictory observations exist concerning the mode of cell death induced by taxol. Some evidence indicates that this drug causes damage leading to mitotic catastrophe – the type of cell death accompanied by the appearance of giant, multinucleated cells with non-condensed chromosomes (Castedo, et al., 2002; Ricci and Zong, 2006; Roninson, et al., 2001). While others claim that taxol induces cell death with typical signs of apoptosis (Bhalla, et al., 1993; Liu, et al., 1994; Yeung, et al., 1999), or necrosis (Michalakis, et al., 2005; Yeung, et al., 1999) depending on the dose of the drug. However, the reason behind the variation in response to taxol is unclear and it is interesting to further elucidate the underlying mechanism. In the present investigation, at 100 nM concentration of taxol or nocodazole, cells showed distinct phenotypes either leading to apoptosis or polyploidy (>4N DNA content) (Figure 9 & Figure 10). Although most of the cell lines treated with taxol induced apoptosis, some are resistant. Interestingly, Burkitt lymphomas resistant to taxol-induced apoptosis showed enhanced polyploidy; whereas cell lines sensititve to apoptosis exhibited limited or no polyploidy. This gives us a clear picture of an inverse correlation between apoptosis and polyploidy upon taxol treatment. Another microtubule inhibitor, nocodazole, which functions by destabilizing microtubules, induces apoptosis similar to that of taxol treatment. This is in line with other reports that nocodazole induces apoptosis in a variety of cell types, for instance, in CLL cells (Beswick, et al., 2006; Verdoodt, et al., 1999) and Colon carcinoma cells (Zhang, et al., 2002). Moreover, a similar pattern of inverse correlation of apoptosis and polyploidy observed upon taxol treatment was also observed with nocodazole treatment.

As mentioned in many reports, taxol exerts effects mainly through regulation of microtubular dynamics that subsequently leads to G2/M arrest, which is usually followed by apoptosis or necrosis (Abal, et al., 2003). In concordance with this, it is clear that

microtubule inhibitors taxol and nocodazole induces arrest at the G2/M phases of the cell cycle in Burkitt lymphoma. More significantly, taxol promotes the accumulation of cyclin B1 and induces the *in vitro* histone H1 kinase activity of CDK1, which triggers G2/M transition and mitotic arrest preceding apoptosis. This histone H1 kinase activity of CDK1 is considered as mitotic marker (Ibrado, et al., 1998). However, there are other reports showing that cells undergoing mitosis are positive for MPM2, a well-established mitosis-specific phospho-epitope and mitotic marker (Andreassen and Margolis, 1994; Davis, et al., 1983). Considering MPM2 as a mitotic marker, all the Burkitt lymphoma cell lines studied were positive for MPM2, signifying mitotic accumulation upon exposure to taxol or nocodazole (Figure 13). Following mitotic arrest, cells undergo apoptosis. However, apoptosis resistant cells show persistant mitotic arrest for prolonged time periods and interestingly, a major portion of mitotically arrested cells are polyploid, which might be a consequence of endoreplication (Figure 14).

This feature of polyploidization is considered as a hallmark of mitotic catastrophe. There are diverse descriptions of mitotic catastrophe from earlier studies. Initially, mitotic catastrophe was associated with incomplete DNA synthesis and premature chromosome condensation (Ianzini and Mackey, 1998; Mackey, et al., 1988) with features in common with apoptosis. Other authors define it as an aberrant form of mitosis associated with the formation of multinucleate giant cells that are temporarily viable but reproductively dead (King and Cidlowski, 1995; Miranda, et al., 1996). Some others state that, mitotic catastrophe is pre-determined in G2 and characterized by an abortive shortcut into metaphase arrest (Kondo, 1995; Rhind and Russell, 1998). Mitotic failure often manifests with micronucleation (Abend, et al., 1996; Heddle and Carrano, 1977) and nuclear segmentation (Dini, et al., 1996) and this in turn is associated with mitosis restitution into interphase polyploid cells (Nagl, 1990). Similar results have been described by others where giant polyploid cells were seen upon treatment of cancer cells with different stimuli, including etoposide (Rello-Varona, et al., 2006), doxorubicin (Eom, et al., 2005), heat shock (Nakahata, et al., 2002) and ionizing radiation (Kakizaki, et al., 2006). So far, only limited studies have been reported about this mode of cell death induced by taxol (Jordan, et al., 1996; Michalakis, et al., 2005; Xiao, et al., 2005). However, it is more apparent from our results (Figure 8 - Figure 10) that the microtubule inhibitors taxol and nocodazole induce polyploidy which might be a consequence of failed apoptosis. There are various reports and

predictions about when polyploidy is triggered during the cell cycle. Polyploidy, which corresponds to a change to an exact multiple of the haploid number of chromosomes (hyperploidy), can be induced via two main routes: rereplication of the DNA in the absence of an intervening mitosis or in the absence of a functional spindle or premature exit from mitosis to the next G_1 phase without having completed chromatid migration to the poles (Kirsch-Volders, et al., 1998). Inhibition of histone deacetylase activity (Shin, et al., 2003) or inhibition of protein kinases in mitotically arrested cells by use of staurosporine triggers premature mitotic exit and polyploidy (Hall, et al., 1996). There are other evidences showing that loss of APC (anaphase promoting complex) leads to chromosomal instability as a result of compromised mitotic spindle checkpoint (Tighe, et al., 2004). Interestingly, data from Figure 14B demonstrates that most of the cells stained for MPM2 are polyploid, which indicates that the possible trigger for polyploidization originates during mitosis.

Vincristine, another microtubule inhibitor interfering with microtubule dynamics, has been used widely in anticancer therapy for more than 30 years (Gidding, et al., 1999). It has a wide range of clinical applications and continues to be a key drug in the combination chemotherapy for childhood acute lymphoblastic leukemia (ALL) and non Hodgkin's lymphoma (NHL) [reviewed in (Gidding, et al., 1999)]. Since the mode of action is not well established, it is interesting to investigate the mechanism involved in cell death induced by vincristine. As shown in Figure 12A, vincristine induces apoptosis to a similar extent in Burkitt lymphomas as observed with taxol or nocodazole in apoptosis sensitive cell lines. Burkitt lymphomas resistant to taxol or nocodazole were also found to be resistant to vincristine treatment; probably the same factors are responsible for the distinct features including apoptosis resistance and sensitivity. Additionally, vincristine treated cells also showed prolonged mitotic arrest in apoptosis resistant but not in sensitive cell lines. Of note and contrary to the effect of taxol or nocodazole, there was no polyploidy induction observed in apoptosis resistant cells (Figure 12B). This established that vincristine treatment specifically fails to induce polyploidization in apoptosis resistant cell lines. This clearly conveys the existence of different signalling mechanisms upon vincristine treatment as compared to that of taxol or nocodazole treatment. Conversely, there are opposing evidences obtained in sarcoma cells undergoing both DNA fragmentation and polyploidy in response to vincristine (Mujagic, et al., 1983). Thus, absence of polyploidy induction in Burkitt lymphomas with vincristine treatment is an interesting aspect. However, further

validatation of mechanistic details would be benificial in understanding the cancer response to microtubule-targeted therapies.

The microtubule inhibitors taxol and nocodazole induce two distinct features in Burkitt lymphomas, one feature leading to cell death via apoptosis and the other feature showing prolonged mitotic arrest and polyploidization. However, the consequence of microtubule inhibitors induced mitotic arrest and polyploidy in resistant Burkitt lymphomas is unclear. It is probable that apoptosis resistant cells undergo delayed apoptosis or die by other means of cell death. Although most of the apoptosis resistant cells are reproductively dead with loss of proliferative ability upon taxol, they are viable with intact plasma membrane (Figure 15). In addition, the effect of taxol seems to be irreversible, where the cells failed to recover after 3 days of drug removal (Figure 16). Nevertheless, it is important to consider the possibility that cells may take a longer duration to recover from taxol effect or subjected to delayed apoptosis. This might impact on the clonogenic survival and lead to better outgrowth of aneuploid subclones.

Interestingly, there are other evidences that generation of polyploid gaint cells provides a survival mechanism by a complex somatic reduction process involving meiotic-like bouquets. This has been reported recently for some Burkitt lymphoma cell lines after high doses of irradiation (Dooley, et al., 1991; Illidge, et al., 2000; MacAuley, et al., 1998). Taken together, it can be predicted that polyploidization is a means of survival mechanism in aggressive Burkitt lymphomas, which is supported by the evidence that aneuploid tumors are associated with high-grade invasive tumors and poor prognosis (Giaretti, 1994; Sandberg, 1977; Segers, et al., 1994; Verdoodt, et al., 1994).

According to clinical evidence, vincristine is more effective than taxol or nocodazole in treatment of Burkitt lymphomas and the reason behind this is unexplored. Probably, the fact that vincristine fails to induce polyploidization probable cancer cell survival mechanism, might explain its better performance compared to taxol in treating Burkitt lymphomas.

So far the findings discussed are presented in a schematic illustration in Figure 41. In brief, Burkitt lymphomas undergo mitotic arrest followed by either apoptosis with no polyploidy or polyploidy without apoptosis induction in response to microtubule inhibitors. Further analysis of apoptosis resistant cells demonstrated persistant mitotic arrest with most of them displaying a DNA content of more than 4N. Moreover, they showed a loss of proliferative ability and failed to recover for a time period of 72 h after microtubule inhibitor

rescue. These phenotypes of prolonged mitotic arrest, polyploidization and loss of proliferative ability observed in resistant Burkitt lymphomas in response to microtuble inhibitors are characteristics of mitotic catastrophe.

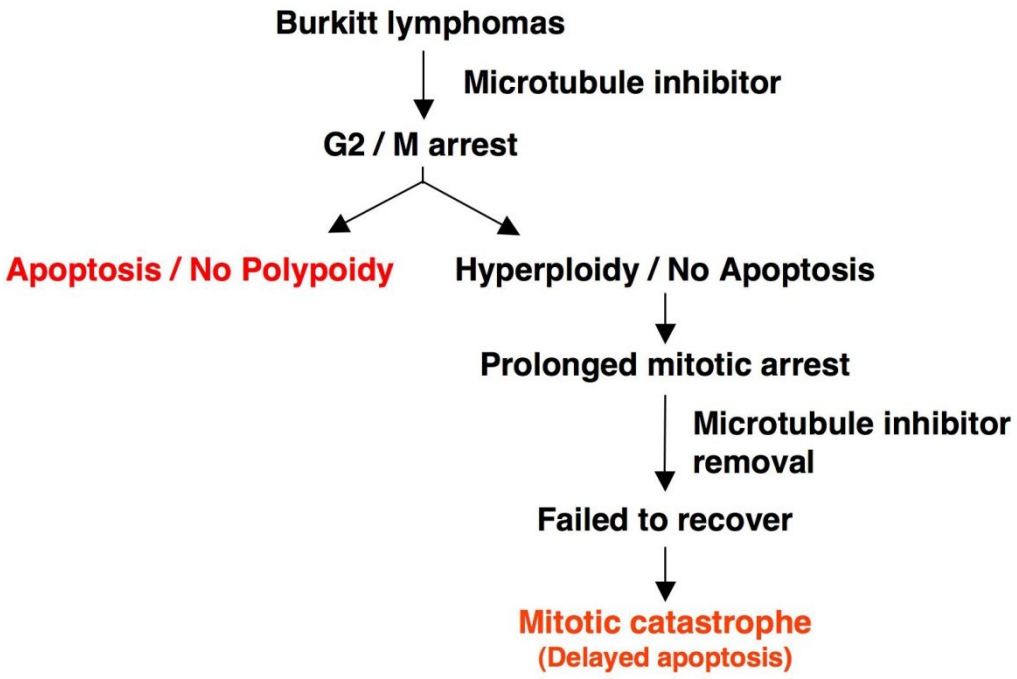

Figure 41: Schematic representation of microtubule inhibitor induced cell death in Burkitt lymphomas: apoptosis or mitotic catastophe. The initial data indicate that stress induced by microtubule inhibitors in Burkitt lymphoma cells stimulated them to either undergo apoptosis without genomic instabilty or to genomic instability i.e. polyploidy without apoptosis. These distinct phenotypes seem to be cell type specific. On the other hand, the effect of microtubule inhibitors on cell viability is irreversible and at the same time apoptotic resistant cells showing hyperploidy fail to induce other ways of cell death like necrosis.

In the subsequent experiments, biochemical aspects associated with apoptosis and mitotic catastrophe were analyzed to understand the rationale behind the sensitivity and resistance to apoptosis. Taxol-induced apoptosis is accompanied by caspase-3, -9 and -8 cleavage in cells sensitive to apoptosis (Figure 17), which is concordant with previous studies, demonstrating that taxol-induced apoptosis involves hierarchical activation of mitochondria, caspases-3, and -8 (Wieder, et al., 2001). In addition, it is well established that taxol-induced cell death depends in part on the activation of caspase-3-like protease activities (Essmann, et al., 2000; Suzuki, et al., 1998). Apoptosis induction and caspase-8 processing by taxol or other drugs such as anthracyclines or nucleoside analogs was

suggested to occur via the CD95/Fas receptor/ligand system (Biswas, et al., 2001; Friesen, et al., 1999; Fulda, et al., 2001; Fulda, et al., 2001; Nomura, et al., 2000). Thus, caspase-8 activation could occur via the death-receptor associated DISC (Muzio, et al., 1998). However, there is evidence showing that caspase-8 activation may also occur in death receptors independent signaling in taxol-induced apoptosis (von Haefen, et al., 2003). In line with this, there are previous studies describing that $cFLIP_L$ may form death receptor independent heterodimers with caspase-8, which leads to weak activation and initial processing of caspase-8 (Krueger, et al., 2001). Another possibility of caspase-8 processing can be explained at least in part by the fact that probably other proteases, such as serine proteases (Huang, et al., 1999) can do the job. Furthermore, inhibition of caspase-8 activity results in impaired DNA fragmentation. Nevertheless, neither the pan-caspase nor the caspase-8-specific inhibitors completely blocked taxol-induced DNA fragmentation (Figure 27 - Figure 30), indicating that caspase-8 independent mechanisms are also at work.

However, apoptosis resistant cells failed to exhibit significant activation or processing of caspase-3 and -9 (Figure 17 - Figure 18), but showed a reduction in caspase-8 proform with sparsely visible cleavage products suggesting slight activation of caspase-8 (Figure 17). Further, amplification of caspase-8 activation or processing is not observed in apoptosis resistant cell lines, probably due to lack of feedback amplification via caspase-3 activity. This is in line with previous reports demonstrating activation of caspase-8 in drug-induced apoptosis via the mitochondrial pathway (Belka, et al., 2000; Engels, et al., 2000; Wesselborg, et al., 1999). This transduction of signals from caspase-8 to mitochondria involves activation of Bax/Bak, which confer an essential gateway for the activation of effector caspases in mitochondrially mediated intrinsic apoptosis pathway (Korsmeyer, et al., 2000). Caspase-8 is activated downstream of caspase-3 during drug-induced apoptosis (Daniel, et al., 2001) including cell death activated by taxol, probably through a feedback loop that seems to also include autoprocessing of the enzyme (Wieder, et al., 2001).

Looking into the regulation of the caspase-8 substrate Bid, a proapoptotic Bcl-2 family member, a reduced proform of Bid was observed in both sets of cells lines exhibiting either apoptosis resistance or sensitivity in response to taxol (Figure 17). This is supported by findings from previous studies that, low amounts of activated caspase-8 are sufficient to process Bid to its active truncated form tBid, thus transducing apoptotic signals from the cytoplasm to the mitochondrial membrane (Gross, et al., 1999; Li, et al., 1998). This further

validates the existence of such a feedback loop amplification of caspase-8 activation. Additionally, the lack of difference in Bid regulation in apoptosis sensitive and resistance cell lines also suggests that, Bid cleavage to tBid has no correlation in imparting apoptosis resistance and induction of polyploidy in response to microtubule inhibitors in Burkitt lymphomas.

There is evidence that *bid* gene is transcriptionally regulated by p53 and may enhance the apoptosis response to specific chemotherapies (Sax, et al., 2002). The importance of apoptosis for p53-mediated tumor suppression has been suggested from correlative studies linking p53 loss to apoptotic defects during the progression of murine and human tumors (Attardi and Jacks, 1999; Bardeesy, et al., 1995), as well as by functional studies demonstrating that strictly anti-apoptotic activities can accelerate tumorigenesis in transgenic mice (Eischen, et al., 2001; Strasser, et al., 1990; Yin, et al., 1997). Furthermore, certain p53 wild type tumors harbor mutations that have been shown to suppress apoptosis downstream of p53 (Soengas, et al., 2001), and some tumor-derived p53 mutants are known to be defective in inducing apoptosis but not cell-cycle arrest (Aurelio, et al., 2000). In the present study, each Burkitt lymphoma cell line has a different p53 status: one cell line has a wild type p53, while others have mutated p53 leading to their stabilization. The status of p53 neither shows any direct correlation on the sensitivity of the cell lines to apoptosis induction nor on polyploidy induction (Figure 19). This implies that taxol induced apoptosis is p53 independent. This is in line with other reports demonstrating that taxol induce apoptosis independently of p53 (Woods, et al., 1995). Reduced levels of p53 correlated with increased G2/M phase arrest, micronucleation, and p53-independent taxol-induced apoptosis (Wahl, et al., 1996). (Giannakakou, et al., 2000) proposed that functional microtubules and the dynein motor protein participate in transport of p53 and facilitate its accumulation in the nucleus after DNA damage. Disruption of normal microtubule dynamics, whether by stabilization with taxol or depolymerization with vincristine, compromises microtubule function. Both of these drug treatments impair nuclear accumulation of p53 after DNA damage (Michael and Oren, 2003). This information in part can explain the lack of p53 stabilization in BL2 cells with endogeneous wild type p53 besides reduction in stable mutant p53 levels in other cell lines, probably through disruption of microtubule/p53 association leading to p53 exposure to proteosomal degradation in response to taxol. Nevertheless, p53 mutation may add to the advantage of deregulated apoptosis in induction of polyploidy,

which is yet to be investigated.

As Bid was found to be regulated in response to microtubule targeted drugs in our hand, it is interesting to check the downstream targets of Bid. There are evidences showing that tBid preferentially activates mitochondria to release cytochrome c in a Bax/Bak-dependent manner (Korsmeyer, et al., 2000; Zamzami, et al., 2000). Nevertheless, several groups have demonstrated that mitochondria can be activated either by Bax or Bak-dependent mechanisms (Gillissen, et al., 2003; Gillissen, et al., 2007; Hemmati, et al., 2002; Radetzki, et al., 2002; von Haefen, et al., 2002; Wendt, et al., 2005). Bak and Bax are generally assumed to substitute for each other, since deficiency of both genes is required to render cells resistant to a number of proapoptotic agents (Janssen, et al., 2007; Wei, et al., 2001). Another group has demonstrated, that lack of bax promotes survival of tetraploid cells at least as efficiently as the p53 or p21 knockout (Castedo, et al., 2006). In the present study, most resistant Burkitt lymphomas lack functional Bax and show weak expression of Bak protein whereas sensitive cell lines are Bax/Bak proficient. This implies that probably lack of functional Bax/Bak confers resistance against taxol-induced apoptosis and in turn leads to induction of polyploidy in Burkitt lymphomas (Figure 20). This also delineates that apoptosis induction upon spindle toxin exposure acts as a fail-safe mechanism to prevent accumulation of aneuploid cells. This seems to be achievied by p53 independent signalling. Similar observations were made here in FDM (factor dependent monocyte) cell lines where double knockouts for Bax and Bak failed to induce apoptosis, and, these cells were triggered for polyploidization (Figure 24). This indicates that Bax/Bak play a crucial role in imparting resistance to apoptosis induction and induction of polyploidy. This is not the case with individual knockouts of Bax or Bak proving their functional redundancy in taxol-induced apoptosis (Figure 24).

Apart from evidences that Bid plays a role in taxol induced cell death, many studies have shown that cells undergo taxol-induced cell death when the mitochondrial apoptosis pathway is activated (Torres and Horwitz, 1998; von Haefen, et al., 2003; Woods, et al., 1995). The regulators of this mitochondrial apoptosis pathway are the BH3-only proteins (Bouillet and Strasser, 2002; Willis and Adams, 2005). Specifically, the BH3-only protein Bim has been shown to play a major role in taxol-induced cell death (Li, et al., 2005; Li, et al., 2007; O'Connor, et al., 1998; Sunters, et al., 2003; Tan, et al., 2005). Depletion of Bim in *in vitro* cell culture models (Li, et al., 2005; Li, et al., 2007; Sunters, et al., 2003) as well as, in *in*

vivo bim−/− mouse models, delays taxol-mediated apoptosis (Tan, et al., 2005). Bim is also known to play an important role in cell death caused by growth factor deprivation in various cell types including neuronal and hematopoietic cells (Dijkers, et al., 2000; Putcha, et al., 2001). However, there is also contrary report demonstrating that Bim is not absolutely required for taxol-induced cytotoxicity (Czernick, et al., 2009). According to the present findings, an essential role of Bim in taxol induced cell death is debatable. Moreover, expression of Bim is highly variable in Burkitt lymphomas with several cell lines being void Bim expression. Another BH3-only protein, Puma, was also shown to be unregulated in apoptosis induced by taxol (Wang, et al., 2007). The present study shows that the pro-apoptotic BH3-only proteins like Bim, Puma, Noxa and Nbk seem to have no role in taxol induced apoptosis in Burkitt lymphomas except for Bid. Instead they are downregulated upon taxol treatment, which might be the cell's counter action against cell death induction (Figure 21). However, the mechanism behind this is unclear and further studies are necessary to understand the mechanism and implications of their downregulation. The genetic knockout of Bim, Puma, Noxa or Bid, respectively in FDM cells, failed to inhibit apoptosis or to induce polyploidy upon taxol treatment (Figure 26). This supports in part the observations made with Burkitt lymphoma cells and also implies the possibility of BH3-only proteins being functionally redundant.

Mcl-1, an anti-apoptotic protein is highly expressed in hematopoietic cells and in leukemias (Andersen, et al., 2005; Chetoui, et al., 2008; Petlickovski, et al., 2005; Sorensen, et al., 2006). Mcl-1 protects against apoptosis and silencing it by a shRNA approach sensitizes HeLa cells to TRAIL-induced apoptosis (Clohessy, et al., 2006) and malignant melanoma cells to Fas-mediated apoptosis (Chetoui, et al., 2008). Genetic studies with the use of a conditional knockout approach also revealed that *Mcl-1* is required both in early lymphoid development and in the maintenance of mature B and T lymphocytes, which are rapidly lost when Mcl-1 is deleted (Opferman, et al., 2003). There is evidence for predominant expression of Bcl-x_L by malignant cells in various types of malignant lymphomas (Xerri, et al., 1996). Additional studies have demonstrated that expression of Bcl-x_L has been largely associated with their drug resistance (Heere-Ress, et al., 2002; Jansen, et al., 1998; Jansen, et al., 2000). Overexpression of Bcl-x_L inhibits taxol-mediated apoptosis (Ibrado, et al., 1997; von Haefen, et al., 2003). There are other evidences showing that taxol downregulates the expression of Bcl-x_L and Mcl-1 (Jazirehi and Bonavida, 2004). Yet, it is

interesting to study how this signalling is carried out on a comparative basis in Burkitt lymphomas and in relation to apoptosis and mitotic catastrophe. The Burkitt lymphoma cell lines used in the present study express Bcl-x_L and Mcl-1; only Mcl-1 expression was decreased in all the cell lines studied after taxol treatment (Figure 22), suggesting that regulation of this protein in different Burkitt lymphomas seems to be similar. However, Bcl-x_L demonstrated downregulation upon taxol exposure only in some cell lines implying the distinct regulation and cell type specificity (Figure 22). There are several mechanisms associated with Mcl-1 regulation. Mcl-1 is phosphorylated at Ser121 and Thr163 by JNK in response to stimulation with H_2O_2, and transfection of unphosphorylatable Mcl-1 resulted in an enhanced anti- apoptotic activity in response to stimulation with H_2O_2 (Inoshita, et al., 2002). Other evidences demonstrated that ERK activation was necessary for the increase in Mcl-1, as inhibition of ERK phosphorylation prevented the increase in Mcl-1 expression and caused rapid cell death by apoptosis (Townsend, et al., 1998). Nevertheless, okadaic acid and taxol induce ERK-independent MCL1 phosphorylation at additional discrete sites in BL41-3 cells (De Biasio, et al., 2007; Domina, et al., 2004). Nocodazole induced phosphorylation of Mcl-1 at ser-64 prominently in the G2/M phase of the cell cycle, which would diminish the anti-apoptotic function of Mcl-1 (Kobayashi, et al., 2007). Mcl-1 is readily downregulated in response to certain death stimuli. The short half-life of Mcl-1 is attributed to constitutive polyubiquitination and subsequent degradation of Mcl-1 by the proteasome, which is a prerequisite for induction of apoptosis after UV irradiation (Nijhawan et al., 2003). Polyubiquitination of Mcl-1 is catalyzed, e.g., by Mule/ARF-BP1, a novel E3 ubiquitin ligase that binds to Mcl-1 via a BH3 domain and marks Mcl-1 for proteasomal degradation (Zhong, et al., 2005) and β-TrCP (Ding, et al., 2007). Mcl-1 was shown as a new substrate for caspases during induction of apoptosis (Herrant, et al., 2004; Weng, et al., 2005). Conversely, Burkitt lymphomas that are resistant to apoptosis induced by taxol also showed Mcl-1 downregulation but failed to activate caspases (Figure 22 and Figure 18). The inhibition of the mitochondrial death pathway by Mcl-1 could proceed through several mechanisms. In this context, it was recently shown that Mcl-1 can interact with tBid and inhibits its ability to activate the mitochondrial death pathway downstream of death receptors (Clohessy, et al., 2006). In summary, these results suggests that Mcl-1 is regulated possibly through ERK or JNK signalling, either leading to a loss of interaction with tBid, thereby activating Bax and/or Bak or by unleashing the direct sequestration of Bax and Bak for further activation.

The only evidence, which distinguished here between apoptosis sensitivity and resistance, is based on the availabilty of Bax/Bak protein. Yet, it is not clear whether Bax and Bax proficiency or the downstream effector molecules act as a connecting link between apoptosis and mitotic catastophe. There is evidence that p53, Bax or p21 KO cells showed reduced cell death and increased hyperploid viability. Besides, pan-caspase inhibitor failed to enhance the polyploidy in nocodazole treated HCT116 cells. Nevertheless, tetraploid cells exhibited an enhanced rate of spontaneous apoptosis that could be suppressed by inhibition of p53 or by knockdown of proapoptotic p53 target genes such as *puma/bbc3*, *gadd45a* and ferredoxin reductase (Castedo, et al., 2006). In line with this, we earlier mentioned the probable role of Bax in polyploidy induction. Besides, inhibition of caspase activity leads to reduced apoptosis in Burkitt lymphomas treated with microtubule inhibitors, which conversely showed enhanced polyploidy (Figure 27 -Figure 28).

However, this inverse correlation between reduced apoptosis and enhanced polyploidy upon pan-caspase inhibition is observed only in response to taxol, nocodazole but not to vincristine treatment (Figure 29). These results confirm the earlier evidence that vincristine has a different mechanism of cell death induction, where it, as shown here, prevents mitotic catastrophe. Hematological malignancies, specifically Burkitt lymphomas, are known to respond even better than solid tumors to vincristine than taxol treatment that is not a drug of choice in clinical lymphoma therapy. Cancers are usually associated with genomic instability, which may favor the generation of more aggressive tumor cells with a reduced propensity for undergoing apoptosis, Thus, absence of genomic instability in vincristine therapy in spite of failed apoptosis would probably justify its efficiency as a better treatment option. The fact that vincristine impedes development of aneuploidy appears to be linked to the beneficial impact of vincristine in lymphoma therapy.

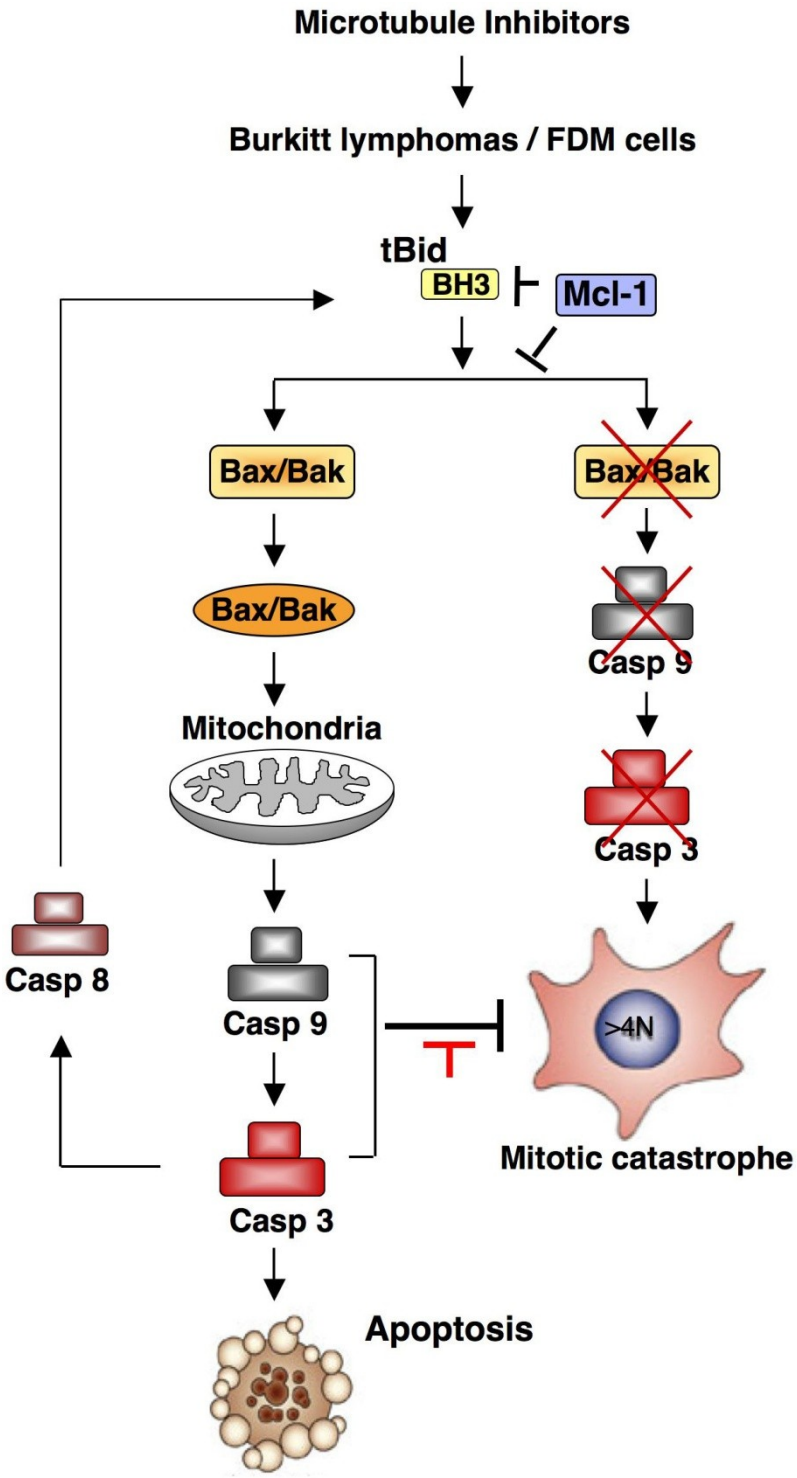

Figure 42: Model illustrating cell death induced by microtubule inhibitors in Burkitt lymphoma cells based on results from this study and litrature. Mcl-1 down-regulation and Bid cleavage lead to the release of Bax and Bak for further triggering of apoptosis signaling cascade through caspase 9, 3 and 8 activation following DNA fragmentation. Conversely lack of Bax and Bak leads to polyploidy showing no sign of apoptosis. In addition inhibiting caspase activity also drives cells in to polyploidy.

Collectively, the data supports a model shown in Figure 42 in which, depending on the proficiency of Bax/ Bak, Burkitt lymphomas induced apoptosis or mitotic catastrophe in response to microtubule inhibitors. Although they induce distinct responses, they showed commonality in downregulation of Mcl-1 and cleavage of Bid. Apoptosis sensitive cells display a hierarichal activation of caspase-8, -9 and -3 leading to execution of apoptosis. Amplification of caspase-8 activation is known to occur in a feedback loop besides autoprocessing (Wieder, et al., 2001). However, the trigger for polyploidization seems to happen downstream of Bax/ Bak. Inhibition of pancaspase activity lead to enhanced polyploidy in apoptotic sensitive cell lines. Nonetheless, vincristine failed to induce polyploidy despite inhibition of caspase activity.

Interestingly, there are other studies showing caspase-dependent cleavage of mitotic checkpoint proteins, Bub1 and BubR1 leading to cell death, in response to microtubule inhibitor exposure. Mutation of caspase cleavage sites in BubR1 leads to prolonged mitotic arrest and increased aneuploidy (Baek, et al., 2005; Kim, et al., 2005). Overexpression of BubR1 predicted tumor recurrence and disease progression in bladder carcinomas (Yamamoto, et al., 2007). In line with these finding, CDC25A phosphatase, a cell cycle regulator acting at the G2/M transition is also cleaved by caspases generating a catalytically active C-terminal fragment. This catalytically active C-terminal fragment reduced the inhibitory phosphorylation of the CDC25A substrate cyclin-dependent kinase 2 (CDK2) on Tyr15 in non-genotoxic apoptotic induction (Mazars, et al., 2009). These data, taken together, infere that caspases play a crucial role not only in the execution of apoptosis but also have a pivotal role in cell cycle check point control and prevention of aneuploidy.

Microtubule-specific drugs elicit the spindle checkpoint through mechanisms that monitor correct spindle formation and tension. In normal cells, this control system preserves genomic integrity by ensuring, that the correct number of chromosomes is distributed to daughter cells. The spindle checkpoint blocks progression into anaphase until all chromosomes have completely aligned at the metaphase plate (Kops, et al., 2005). Various spindle checkpoint proteins, BubR1, Bub1, Bub3, Mad1, Mad2, and Plk1 are proposed as components of a sensorial system that monitors lack of tension or attachment between the kinetochore and microtubules of the mitotic spindle (Hardwick, 2005). Thereby, playing a multifunctional role in mitotic progression (Weaver, et al., 2003). There are convincing evidences from cell culture experiments and animal models that targeted inhibition of genes

involved in controlling cell proliferation and apoptosis combined with various anticancer drugs is a promising approach for cancer therapy (Kim, et al., 2004; Kim, et al., 2004; Lee, et al., 2004; Sonnemann, et al., 2004; Zhang, et al., 2003).

PLK1 has gained attention in recent years, because it has multiple functions during the cell cycle and is known to be overexpressed in approx. 80% of human tumors of diverse origins. Overexpression of PLK1 promotes neoplastic transformation of human cells (Park, et al., 2009; Strebhardt and Ullrich, 2006). Elevated levels of PLK1 expression correlate with poor prognosis for a wide range of human cancers, such as non-small-cell lung cancer, oropharyngeal carcinoma, esophageal carcinoma, melanoma, colorectal cancer, hepatoblastoma, and non-Hodgkin lymphoma (Strebhardt and Ullrich, 2006). PLK1 knockdown studies in cancer cells revealed increases in G_2/M arrested cells up to 5-fold compared with controls followed by apoptosis induction (Spankuch, et al., 2006). Numerous investigations have now established that PLK1 is a prime target candidate for drug development in proliferative diseases such as cancer (Eckerdt, et al., 2005).

Although there were many studies involving PLK1 knock down in various cancer cells, its functional mechanism is not well understood. Therefore, Burkitt lymphoma cells with conditional overexpression of Polo box domain of PLK1 (PLK1-PBD), kinase domain (PLK1-KD) and PLK1, respectively were created to understand its relevance in cell death induction and mechanisms involved. The Polo box domain of PLK1 confers molecular alterations of PLK1 inhibition by tethering away endogenous PLK1 from its original subcellular localization sites and substrates. Because this approach has successfully been used to validate the PBD function (Hanisch, et al., 2006), we also relied in our studies on this mode of PLK1 interference. The ectopic expression of PLK1 or PLK1-KD or PLK1-PBD did not demonstrate enhanced apoptosis compared to control (Figure 32). In concordance to earlier reports mentioned (Spankuch, et al., 2006), cell cycle analysis demonstrated acumulation of PLK1-PBD overexpressing cells in the G2/M phase of the cell cycle, which was limited in PLK1 and PLK1-KD overexpressing cells (Figure 33). There are evidences describing PLK1 regulation in G2 phase of cell cycle upon genotoxic stress, but its role in eliciting G2 checkpoint arrest is not clear (Bassermann, et al., 2008). MPM2 staining (Figure 29) showed that, only a portion of the G2/M phase population is in the M-phase implying that, PLK1 deregulation triggers checkpoint activation not only at M-phase but also at the G2-phase of cell cycle. Though PLK1-PBD overexpression failed to induce high rates of apoptosis in Burkitt lymphoma cell

lines, it resulted in an antiproliferative effect and induction of necrosis in Burkitt lymphomas (Figure 37). These results are in part supported by earlier evidences that Plk1-PBD only triggers, a growth delay in RKO cells and a growth delay plus the occurrence of an increased number of binucleated cells in the PC-3 cell line without induction of cell death. Besides, inhibition of PLK1 by PLK1 kinase inhibitor resulted in mitotic accumulation followed by apoptosis in these cell lines. Nonetheless, the authors also showed that the cellular phenotype observed after ectopic expression of the PBD in U2OS and HeLa cells contrasts the effects observed in RKO and PC-3 cells, where cells demonstrated significant mitotic accumulation accompanied with subsequent onset of programmed cell death (Fink, et al., 2007). Evidently, most studies related to PLK1 functions are conducted in U2OS and HeLa cells (Hanisch, et al., 2006; Kang, et al., 2006; Lee, et al., 1998; Lowery, et al., 2005).

In the present study, the Burkitt lymphoma cells examined showed similar features of an antiproliferative effect, induction of G2/M arrest and slight induction of apoptosis upon functional abrogation of PLK1 with PBD overexpression (Figure 35). Among the Burkitt lymphomas investigated, DG75 and CA46 cell lines showed less apoptosis induction compared to the BL41 cell line. Interestingly, only DG75 and CA46 cell lines showed induction of polyploidy, displaying the inverse relationship between induction of apoptosis and polyploidy (Figure 36). This can be attributed to the loss of functional Bax and a weak expression of Bak, which might enhance their affinity for induction of polyploidy in consequence of their failure to die by apoptosis. Thus, BL41 cells failed to develop polyploidy, which can be explained by their Bax/Bak proficiency (Figure 35). In subsequent experiments with PLK1-PBD overexpression, cervical carcinoma HeLa cells underwent apoptosis in a caspase dependent manner (Figure 38), which is in concordant to previous reports of (Fink, et al., 2007). Inhibition of caspases in addition to functional abrogation of PLK1 in apoptosis sensitive HeLa cells leads to reduced apoptosis. Additional treatment of PLK1 overexpressing cells with microtubule inhibitors showed similar levels of apoptosis inhibition upon inhibition of caspases indicating that both PLK1 interference and taxanes trigger caspase dependent death pathways. Nevertheless, only cells with functional abrogation of PLK1 displayed enhanced polyploidy irrespective of taxol treatment upon caspase inhibition (Figure 39). Further, in line with other's (Hanisch, et al., 2006), immunofluorescence studies in HeLa and BJAB cells showed that the PBD induce checkpoint-dependent mitotic arrest characterized by aberrant spindle formation leading to monopolar,

multipolar or bipolar spindles with lagging chromosomes. Consequently these events lead to the chromosomal instability followed by the cell death. While in the case of failed apoptosis, these events would result in polyploidization. Taken together, it is evident that the PLK1 inhibition and taxol work in the same pathway. Caspases play a pivotal role in cell cycle checkpoint induced apoptosis and act as a connecting link between apoptosis and mitotic catastrophe. These data further strengthen the inverse relationship between induction of apoptosis and polyploidy.

Sensitivity to taxol was previously shown to correlate with decreased BubR1 protein expression in human cancer cell lines, including those from breast and ovarian cancer (Ditchfield, et al., 2003; Lee, et al., 2004). This observation is consistent with an earlier report of increased drug sensitivity after knockdown of BubR1 (Ditchfield, et al., 2003). Thus, drug-sensitive cells were found to lack one or more components of the mitotic spindle checkpoint signalling machinery. Making the spindle checkpoint less robust would possibly make cancer cells more sensitive to drugs that disrupt microtubule dynamics. Thus, it might be that dominant negative inhibition of PLK1 at the kinetochores may stabilize microtubule attachment to kinetochores and contributes to an imbalance of the spindle checkpoint, causing increased sensitivity to microtubule drugs such as taxol, vincristine and nocodazole. In concordance with this, a combination of PLK1 knockdown through antisense inhibitors against PLK1 and taxol treatment have been shown to cause synergistic effects in breast cancer cells (Spankuch, et al., 2006). In the present study, deregulation of PLK1 function and microtubule inhibitor treatment failed, however, to show synergistic effects in induction of apoptosis, which might be attributed to cell type specificity (Figure 38 - Figure 39). Mitotic spindle inhibitors and / or abrogation of mitotic checkpoint regulator PLK1 probably have the same downstream effector proteins being regulated, and as a result, lack synergism.

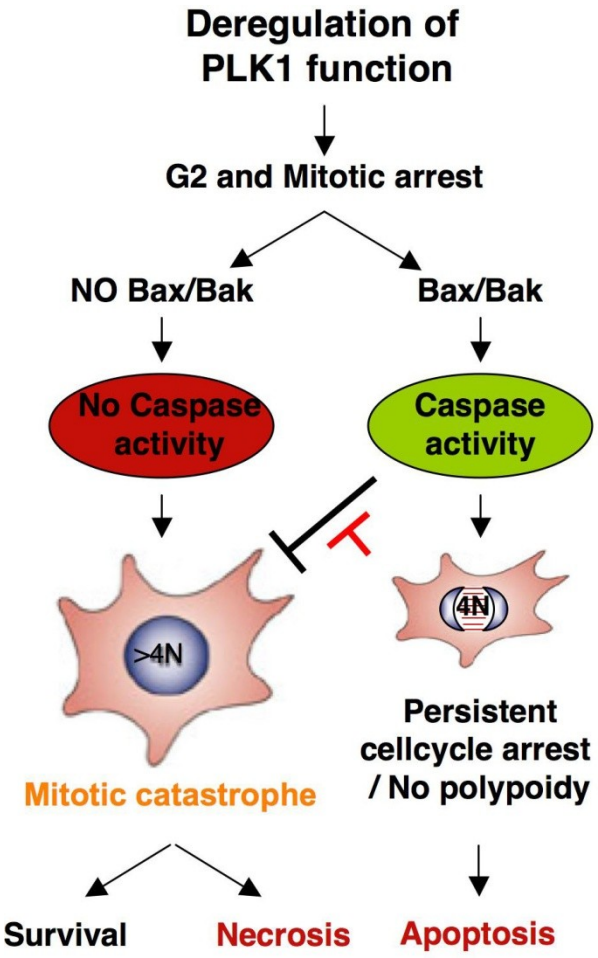

Figure 43: Model illustrating cell death induced by abrogation of cell cycle checkpoint regulator PLK1 in Burkitt lymphoma cells based on results from this study. Displacing the endogeneous PLK1 by PBD overexpression leads to the cell cyle arrest at both G2 and mitotic phase. Further depending on Bax and Bak proficiency and caspase activity, cells either undergo mitotic catastophe or cell death via necrosis or apoptosis depending on the differences in cell type.

In conclusion, this study yields various interesting results giving broad insight in to the biology of Burkitt lymphomas and their response to abrogation of mitotic spindle dynamics. These findings provide clear experimental evidence of an inverse correlation between apoptosis and mitotic catastrophe in various Burkitt lymphomas in response to microtubule inhibitors. Microtubule inhibitor taxol-induced apoptosis proceeds via a caspases-8/-3-driven mitochondrial amplification loop and Mcl-1, Bid, Bax and Bax as upstream regulators. Failed apoptotic signal transduction due to Bax/ Bak deficiency or effector caspase inhibition leads to polploidy with more than 4N DNA content. However, this is not the consequence of vincristine treatment, which failed to induce polyploidy in the absence of Bax/Bak and

caspase activity. Abrogation of PLK1 function induces checkpoint activation further leading to cell death in a caspase dependent manner and lack of caspase activity leads to genomic instability. Our data clearly delineate that intrinsic resistance and genomic instabilty of Burkitt lymphomas against mitotic spindle inhibitors or abrogation of mitotic cell cycle checkpoint regulator PLK1 may be linked to the status of Bax/Bak and caspase activity but not the p53 pathway. These results provide novel insights for better disease prognosis by considering targets to induce Bax/Bak independent caspase activation in addition to microtubule inhibitors. As a perspective, it would also be of great importance to identify novel caspase substrates and proteins associated in triggering endoreduplication leading to genomic instability and aneuploidy.

5 Materials and methods

5.1 Materials

5.1.1 Cell lines

Mammalian cell lines	Source or reference
Burkitt lymphoma cell lines	Georg W. Bornkamm Helmholtz Zentrum München Germany
A1	
Akata	
BJAB	Simone Fulda University of Ulm Germany
BJAB BclXL	
BL like P493-6	Georg W. Bornkamm Helmholtz Zentrum München Germany
BL2	
BL29	
BL41	
BL41 B95-8	
BL41P3HR1	
BL70	
CA46	
DG75	
HH514	
Mutu1	
Factor dependent monocyte cell lines	Paul Ekert Royal Children's Hospital Melbourne Australia
FDM *Bak-/-*	
FDM *Bax-/-*	
FDM *Bax/Bak-/-*	
FDM *Bid-/-*	
FDM *Bim-/-*	
FDM *Noxa-/-*	
FDM *Puma-/-*	
FDM WT	
Human cervical epithelial cell line	Scherer et al.
HeLa	

5.1.2 Genetically modified cell lines

Transfectants	Description
BJAB-Mock	BJAB cells harboring pRTS1 vector with tet-inducible promoter
BJAB-PLK1	BJAB cells harboring *PLK1* ORF with tet-inducible promoter
BJAB-PBD	BJAB cells harboring Polo-box-domain with tet-inducible promoter
BJAB-KD	BJAB cells harboring Kinase domain with tet-inducible promoter
BL41-Mock	BL41 cells harboring pRTS1 vector with tet-inducible promoter
BL41-PLK1	BL41 cells harboring *PLK1* ORF with tet-inducible promoter
BL41-PBD	BL41 cells harboring Polo-box-domain with tet-inducible promoter
BL41-KD	BL41 cells harboring Kinase domain with tet-inducible promoter
CA46-Mock	CA46 cells harboring pRTS1 vector with tet-inducible promoter
CA46-PLK1	CA46 cells harboring *PLK1* ORF with tet-inducible promoter
CA46-PBD	CA46 cells harboring Polo-box-domain with tet-inducible promoter
CA46-KD	CA46 cells harboring Kinase domain with tet-inducible promoter
DG75-Mock	DG75 cells harboring pRTS1 vector with tet-inducible promoter
DG75-PLK1	DG75 cells harboring *PLK1* ORF with tet-inducible promoter
DG75-PBD	DG75 cells harboring Polo-box-domain with tet-inducible promoter
DG75-KD	DG75 cells harboring Kinase domain with tet-inducible promoter
HeLa-Mock	HeLa cells harboring pRTS1 vector with tet-inducible promoter
HeLa-PLK1	HeLa cells harboring *PLK1* ORF with tet-inducible promoter
HeLa-PBD	HeLa cells harboring Polo-box-domain with tet-inducible promoter
HeLa-KD	HeLa cells harboring Kinase domain with tet-inducible promoter

5.1.3 Cell culture media

Cell culture media including RPMI and DMEM were from (GIBCO BRL)

5.1.4 Buffers and solutions

Agarose gel electrophoresis

50X TAE buffer (Stock solution)

242 g	Tris base
57.1 ml	glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)

SDS PAGE sample lysis buffer

0.606 g	Tris –Amino hydroxide
9.242 g	Saccharose
1 ml	TritonX100
2 Pellets	Protease Inhibitor

Made up the volume 100ml with H_2O after adjusting the pH to 7.5

5X SDS gel loading buffer:

300mM	Tris-Cl (pH 6.8)
3%	ß-Mercaptoethanol
50%	Glycerin
0.1% w/v	Bromophenolblue
2% w/v	SDS

1X SDS PAGE running buffer

25 mM	Tris
0.1% w/v	SDS
200 mM	Glycin

Adjusted pH to 8.3

Western blot transfer buffer

10%	Methanol
10 mM	CAPS (pH 11)

10X Ponceau S stain

2 g	Ponceau S
30 g	Trichloroacetetic acid
30 g	Sulfosalicylic acid

Made up the volume to 100 ml with H_2O

10 X PBST

10 X	PBS

2.5 ml Tween-20

Blocking buffer

7-10% Casein-blocking reagent from Roche (#11921673001) in 1X PBST

Western blot stripping buffer

62.5 mM Tris/ HCl pH 6.7

100 mM β-Mercaptoethanol

2% SDS

Protein marker

ColorBurst™ Electrophoresis Marker (Sigma, #C1992)

1X PBSTF

10 ml 10X PBS

10 ml FBS

50 µl Tween-20

Pepsin

0.05 g Pepsin

5 ml 2M HCl

Made up the volume to 100 ml with H_2O

Borax buffer

0.1 M Sodium tetraborate (pH 8.5)

Freezing medium

20% DMSO

80% FBS

LB Broth (Agar)

10 g Bacto-typtone

5 g Bacto-yeast extract

10 g NaCl

20 g Agar (for plates only)

Made up the volume to 1l with H_2O after pH was adjusted to 7.0 with 5N NaOH and sterilized by autoclaving.

5.1.5 Fine chemicals

Chemical	Company and catalog number
Taxol (Paclitaxel)	Bristrol-Myers Squibb GmbH, #7375335
Nocodazole	Sigma, # M1404
Vincristinsulfat	GRY-Pharma GmbH, # 7555221
Hoechst 33342	Sigma, # B2261

5.1.6 Kits used in this study

Kit	Company and catalog number
Western pico supersignal (ECL)	Pierce, # 0034078
BCA™ Protein assay Kit	Pierce, # 23225
QIAquick Gel Extraction Kit	Qiagen # 28704
QIAquick PCR Purification Kit	Qiagen # 28104
PureLink™ Quick Plasmid Miniprep Kit	Invitrogen, # K210011
PureLink™ HiPure Plasmid Maxiprep Kit	Invitrogen, # K2100-02
Green FLICA Caspase 9 Assay Kit	Immunochemistry, # 912
Green FLICA Caspases 3&7 Detection kit	Immunochemistry, # 93

5.1.7 Antibodies

Table 4: List of the primary antibodies employed in this study

Antigen	Species	Dilution for Western Blot	Company and catalog number
Anti-phospho-Ser/Thr-Pro, MPM2	Mouse	1:1000	Upstate, # 05-368
Bak	Rabbit	1:1000	Sigmaaldrich, # 5897
Bax	Rabbit	1:1000	Upstate, # 06-499
Bcl-x	Rabbit	1:1000	BD pharmingen, # 610211

Bid	Rabbit	1:1000	Cell Signalling, # 2002
Bim	Rabbit	1:500	Cell Signalling, # 2819
Caspase-3	Rabbit	1:1000	R & D, # AF835
Caspase-8	Mouse	1:1000	Alexis, # ALX-804-242
Caspase-9	Goat	1:200	R & D, # AF8301
C-Myc (9e10)	Mouse	1:500	Santa cruz, # SC40
C-Myc (A-14)	Rabbit	1:1000	Santa cruz, # SC789
Mcl-1	Rabbit	1:1000	Santa cruz, # SC20679
NBK (N-19)	Goat	1:1000	Santa cruz, # SC1710
Noxa(N-15)	Goat	1:1000	Santa cruz, # SC26917
p53	Mouse	1:500	BD pharmingen, # 554293
PLK1	Mouse	1:1000	Zymed, # 31-7100
Puma(N-19)	Goat	1:1000	Santa cruz, # SC19187
β-actin	Rabbit	1:1000	Sigma, # A2066

Table 5: List of the secondary antibodies employed in this study

Antigen	Dilution for Western blotting	Dilution for Immuno-fluorescence	Company and catalog number
Goat Anti Mouse IgG(H+L)	1:1000	-	Southern Biotech, # 1031-05
Goat Anti Rabbit IgG(H+L)	1:1000	-	Southern Biotech, # 4050-05-147
Rabbit Anti Goat IgG(H+L)	1:1000	-	Southern Biotech, # 6160-05
FlTC Goat Anti Mouse IgG(H+L)		1:200	Jackson immuno research, # 115-095-062

5.1.8 Primers

Forward Primer SfiI: 5’ GGC CTC ACT GGC CAA GCT GGC TAG CGT TT 3’
Reverse Primer SfiI: 5’ GGC CTC ACT GGC CCT CTA GAC TCG AGC 3’

5.1.9 Plasmids and expression constructs

Plasmid Name	Description
pcDNA3.1(+/-)	Invitrogen

pGEM T	Promega, Wisconsin, USA
pRTS1	Georg W. Bornkamm et al.
pBSK-PLK1	Full length PLK1 cDNA cloned in to pBluescript II SK(+/-) vector (provided by Eric. A. Nigg)
pcDNA 3.1/mycC-PBD wt	Polo Box domain of PLK1 cloned in to pcDNA3.1(-) vector (provided by Eric. A. Nigg)
pGEM T_SfiI(+/-)	Shuttle vector contruct was generated by PCR amplification of multiple cloning site of pCDNA3.1(+) using primers flanked with SfiI site, followed by insertion of PCR product in to pGEM T Vector
pcDNA 3.1_PLK1	Full length *PLK1* from pBSK-PLK1 vector was subcloned in to pcDNA 3.1(+) expression vector at EcoR V and Not I sites
pGEM T_SfiI+_PLK1	Full length *PLK1* from pBSK-PLK1 vector was subcloned in to pGEM T SfiI(+) expression vector at HindIII and XbaI sites
pGEM T_SfiI+_PBD	*PBD* from pcDNA 3.1/mycC-PBD wt vector was subcloned in to pGEM T SfiI(+) expression vector at HindIII and XbaI sites
pGEM T_SfiI+_KD	*KD* from pcDNA 3.1/mycC-KD vector was subcloned in to pGEM T_ SfiI(+) expression vector at HindIII and XbaI sites
pRTS1_PLK1	Full length *PLK1* from pGEM T_Sfi I(+)_PLK1 was subcloned in to pRTS1 expression plasmid at SfiI site
pRTS1_PBD	*PBD* from pGEM T_SfiI(+)_PBD was subcloned in to pRTS1 expression plasmid at SfiI site
pRTS1_KD	*KD* from pGEM T_SfiI(+)_KD was subcloned in to pRTS1 expression plasmid at SfiI site

5.1.10 Enzymes

Restriction endonucleases used in this study for molecular cloning were from NEB

*Not*I	*Afl*II	*Hin*dIII	*Nhe*I	*Eco*RI	*Bst*XI	*Apa*LI	*Xba*I
*Eco*RV	*Kpn*I	*Xho*I	*Nde*I	*Bam*HI	*Sfi*I	*Bgl*II	

Other DNA modifying enzymes

Name of enzyme	Company and catalog number
GoTaq® DNA Polymerase (5 U/µl)	Promega, Mannheim, # 9PIM300
Crimson™ Taq DNA Polymerase (5 U/µl)	NEB, Frankfurt am Main, # M0324
T4 DNA Ligase (100 U/µl)	Invitrogen, Karlsruhe, # 15224-017

5.2 Methods

5.2.1 Cell biology methods

5.2.1.1 Culturing of mammalian cells

All mammalian cell lines were maintained at 37°C and 95% humidity in the presence of 5% CO_2. Confluent cultures were subcultured at a density of 1×10^5 cells/ml.

Human Burkitt lymphoma cell lines (BL) and mouse factor dependent monocytes (FDM) were cultivated in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.56 g/l L-glutamine. For FDM cells, 10% IL3 was additionally added to the culture media.

HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high-glucose supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 g/ml streptomycin (all from Gibco, Karlsruhe, Germany). Tet-inducible BL cells and HeLa cells were cultured in media with or without 1 µg/ml doxycyclin.

5.2.1.2 Freezing and thawing procedure

Freezing: Log phase cells were pelleted by centrifugation at 300*g* for 5 min, washed with 1X PBS and resuspended in culture medium and freezing medium in the ratio 1:1 and preserved in cryovial at -80°C for short term storage and in liquid nitrogen for long term storage.

Thawing: Frozen cells were thawed, transferred in to pre-warmed culture medium, pelleted and resuspended in fresh medium in a tissue culture flask.

5.2.1.3 Microtubule inhibitor treatment

Suspension cells were diluted to 4×10^5 cells/ml one day prior to starting an experiment. Cells were seeded with the density of 1×10^5 cells/ml with the desired concentrations of the respective cytostatic drugs. In standard experiments taxol, vincristine and nocodazole were used at a concentration of 100 nM.

5.2.1.4 Transient transfection of BL cells

Plasmids encoding *PLK1*, *PBD* and *KD* were kindly provided by Prof. E. Nigg, and used for further cloning into pRTS-1 plasmid. In general, 6×10^5 B cells were transfected with 10 µg of pRTS-1 DNA by electroporation and expanded in 500 µg/ml or 600 µg/ml hygromycin B for CA46, BL41 or BJAB cells, respectively for at least 2 weeks. Selection was performed either in the presence or in the absence of doxycycline. Since a sub-population of non transfectants were hygromycine B resistant and outgrew the transfectants, cells were partially induced for 12 - 16 h with 1 µl/ml doxycycline and eGFP positive cells were FACS sorted. These cells were used for further experiments.

5.2.1.5 Stable transfection and establishment of single cell clones

Lipofection was carried out as described later in 5.2.1.13.2 and the cells were allowed to recover in 10 ml growth medium for two days at 37°C and 5% CO_2. Cells were counted and plated into 96-well plates in 100 µl at a density of 0.6 viable cells per well. After 5–6 days, 100 µl growth medium was added containing 250 µg/ml hygromycin B for HeLa cells. Replacing 50 µl of the cell supernatant with fresh selection medium weekly, the hygromycin B concentration was slowly increased to a final concentration of 300 µg/ml. Within the next 2 - 4 weeks, hygromycin B resistant cells grewout. The outgrowing cells were regarded as single cell clones, which were confirmed by observing portion of cells for eGFP expression by flow cytometry after induction with doxycycline for 24 h. When an appropriate cell density was reached, cells were expanded successively into 48 and 24 well plates, and finally into 75 ml tissue culture flasks and maintained in culture under hygromycin B selection in the absence of doxycycline.

5.2.1.6 Measurement of cell death

5.2.1.6.1 Propidium iodide uptake

For the determination of cell death, 3×10^4 cells per well were seeded in 96 well microtiter plates and treated for different time periods with microtubule inhibitor. Cell death was assessed by the uptake of propidium iodide (PI) (2 µg/ml) in phosphate-buffered saline (PBS) into nonfixed cells and by subsequent flow cytometric analysis using the FSC-FL3 profile.

Analysis was performed on a FACScalibur (Becton Dickinson; Heidelberg, Germany) using CellQuest analysis software.

5.2.1.6.2 Trypan blue exclusion

Cells were harvested at 300*g* for 5 min and pellet was resuspended in 1X PBS. 10 µl of cells were incubated with 90 µl 0.4% trypan blue solution for 5 min. 10 µl of stained cells was added in a Neubauer counting chamber and counted for the number of viable (unstained) and dead (stained) cells. Average number of unstained cells in each quadrant was calculated, and multiplied by 1×10^5 to determine cells/ml. Blue cells were assumed as dead. At least 100 cells were counted.

5.2.1.7 XTT cell proliferation and viability assay

The cell viability was determined by XTT assay using a Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the manufacturer's protocol. In brief, cells were seeded at a concentration of 4×10^3 cells/well in 100 µl of cultured media into microplates (tissue culture grade, 96 wells, flat bottom). Cells were incubated for 3 days with respective cytostatic drug treatment at 37°C and 5% CO_2. To this 50 µl of XTT labeling mixture was added and incubated for 4 - 24 h at 37°C and 5% CO_2. Spectrophotometrical absorbance of the samples was measured using a microplate (ELISA) reader at 450 nm keeping reference wavelength at 620 nm. In each treatment group, triplicates wells were measured for cell viability.

5.2.1.8 DNA fragmentation and cell cycle analysis

Cells were collected by centrifugation at 300 *g* for 5 min, washed with 1X PBS at 4°C, and fixed with PBS/2% (vol/vol) formaldehyde on ice for 30 min. After fixation, cells were incubated with ice-cold ethanol/PBS (2:1, vol/vol) for 15 min, pelleted, and resuspended in 1X PBS containing 40 µg/ml RNase A. After incubation for 30 min at 37°C, cells were pelleted again and finally resuspended in PBS containing 50 µg/ml propidium iodide. Nuclear DNA fragmentation and polyploidy were determined by flow cytometric quantification of hypodiploid DNA and hyperploidy DNA content. Data were collected and analyzed using a FACScan (Becton Dickinson) equipped with the CELLQuest software. Data are given in

percentage hypodiploidy (subG1) and percentage hyperploidy (>4N DNA content), which reflects the number of apoptotic cells and polyploid cells, respectively.

5.2.1.9 Quantitative DNA cell cycle analysis and cell proliferation assays

Cells were collected by centrifugation at 300 *g* for 5 min, washed with 1X PBS at 4° C, and fixed with ice-cold ethanol/PBS (2:1, vol/vol) for 15 min. Fixed cells were pelleted, and resuspended in 200 µl of pepsin (0.4 mg/ml in 0.1 M HCl). After incubation for 30 minutes at room temperature, cells were pelleted again and resuspended in 200 µl of 2 M HCl. After incubation for 30 min at 37ºC, cells were pelleted and resuspended in 200 µl of borax buffer for 5 min. Cells were pelleted again, washed and finally resuspended in PBS containing 50 µg/ml propidium iodide. Data were collected using a FACScan (Becton Dickinson) equipped with the CELLQuest software and analyzed using Modfit LT software, the most robust and simultaneously easy-to-use software tool for DNA -ploidy /proliferation analysis.

5.2.1.10 Determination of the mitotic index

Cells were collected and resuspended in 70% ethanol overnight at −20°C. Cells were pelleted, washed with 1X PBST and incubated with a phospho-Ser/Thr-Pro MPM2 monoclonal antibody diluted in 1X PBST for 1 h at 4ºC. Further cells were washed and labeled with a FITC-conjugated anti-mouse secondary antibody for 1 h at room temperature in the dark. Cells were then washed twice with 1X PBST and resuspended in a propidium iodide (PI) solution consisting of 50 µg/ml PI and 50 µg/ml RNase A for 15 min. Cells were then sorted and analyzed using FACScan (Becton Dickinson) equipped with the CELLQuest software.

5.2.1.11 Caspase activation assay

Active caspases were detected using FLICA Apoptosis Detection Kits for Caspase-3/7 and Caspase-9 (Immunochemistry Technology, LLC, Bloomington, MN) according to the manufacturer's instructions. This kit employs carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitors of caspases-3/7 (FAM-DEVD-FMK) and -9 (FAM-LEHD-FMK), which are cell-permeable and non-cytotoxic fluorochrome inhibitors. These labeled peptide inhibitors covalently bind to a reactive cysteine residue on the large subunit of the active

caspase heterodimer, inhibiting enzymatic activity and producing green fluorescence. Thus, the green fluorescent signal directly corresponds to the amount of active caspases present in the cell at the time the reagent was added.

In brief, cells cultured in the presence and absence of chemostatic drug for 48 h were harvested, washed in 1X PBS and resuspended in FLICA working solution and allowed to incubate for 1 h at 37ºC, 5% CO_2 in fully humified atmosphere in dark with occational gentle swirlling. Thereafter, cells were washed with 1X PBS and resuspended in 200 µl PBS before determining the fluorescence or Caspase activation with a FACScan flow cytometer.

5.2.1.12 Caspase inhibition assay

Q-VD-OPh (N-(2-Quinolyl)valyl-aspartyl-(2,6-difluorophenoxy)methyl Ketone) is a cell-permeable, irreversible, broad-spectrum caspase inhibitor (Calbiochem). Z-IETD-FMK (Z-IE(OMe)TD(OMe)-FMK) is a potent, cell-permeable, and irreversible inhibitor of caspase-8 (Calbiochem). For inhibiton of pan-caspase activity or caspase-8 activity, cells were incubated with 10 mM Q-VD-OPh or 20 mM Z-IETD-FMK respectively 2 h prior to treatment with microtubule inhibitors. These cells were further analyzed for apoptosis after 72 h.

5.2.1.13 Transient Transfection

5.2.1.13.1 Electroporation

5×10^6 cells in log phase were washed with 1X PBS and resuspended in 400 µl of RPMI media without FBS and pencilline/streptomycine. To this 10 µg of plasmid DNA was added and incubated for 5 min at room temperature. DNA/cell suspension was transferred to an electroporation cuvette (with 0.4 cm interelectrode distance) and electroporated (BioRad gene pulser) at 250 V and 960 µF for 12 sec. The mixture in the cuvette was left for 10 min at room temperature before transfering the cells to T75 flask containing culture media with 10% FBS.

5.2.1.13.2 Lipofection

Plasmid trasfection was carried out using Lipofectamine 2000 reagent according to the manufacturers (Invitrogen) instructions.

5.2.2 Molecular biology methods

5.2.2.1 Construction of shuttle vector pGEM-T_SfiI

Multiple cloning sites from pcDNA3.1+ were PCR amplified using forward and reverse primers flanked with *Sfi*I recognition sites. Primer sequences are reported in materials as Forward Primer SfiI and Reverse primer SfiI. The amplified product was analyzed on 1% agarose gel and purified using PCR gel elution kit (Qiagen). The 135 bp DNA fragment obtained was cloned into pGEM-T Easy vector. Plasmid and insert orientation were confirmed by restriction digestion. Plasmid was named as pGEM-T_SfiI+ (Figure 44) or as pGEM-T_SfiI- depending on the orientation of the insert.

5.2.2.2 Construction of pGEM T_SfiI+_PLK1

Full length ORF of *PLK1* gene (2119 bp) from the bluescript II sk(+/-)-PLK1 (gifted by Prof. E. Nigg) was excised with *Hin*dIII-*Xba*I and subcloned into *Hin*dIII-*Xba*I sites of pGEM-T_SfiI(+) vector. Similarily, Polo Box Domain of *PLK1* gene (831 bp) and Kinase Domain of *PLK1* (1056 bp) from parent plasmid pcDNA3.1/mycC-PBD wt and pcDNA3.1/mycC-aa1-352, respectively (gifted by Prof. E Nigg) were excised with *Hin*dIII-*Xba*I and subcloned into the *Hin*dIII-*Xba*I sites of pGEM-T_SfiI(+) vector (Figure 44). Plasmids with *PLK1*, *PBD* or *KD* were confirmed by restriction digestion. Figure 44A depicts the plasmid map of pGEM T_SfiI(+) with gene of interest.

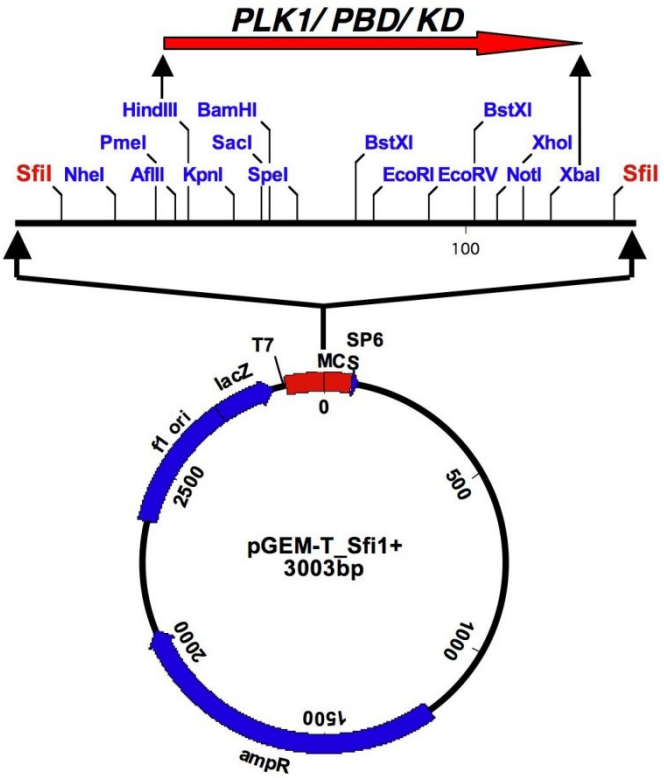

Figure 44: Subcloning of *PLK1* (Polo like kinase 1), *PBD* (polo box domain of *PLK1*), and *KD* (Kinase domain of *PLK1*) in to shuttle vector pGEM-T SfiI+. *PLK1* excised from pBluescript II SK(+/-) and *PBD*, *KD* fragments from pcDNA3.1 3x myc-C plasmids, respectively with *Hin*dIII/ *Xba*I REases and were cloned into pGEM-T_SfiI(+) shuttle vector having same flanking ends.

5.2.2.3 Construction of pRTS-1_PLK1, pRTS-1_PBD and pRTS-1_KD

Full length *PLK1*, Polo Box Domain of *PLK1* gene and Kinase Domain of *PLK1* from shuttle vector pGEM-T_SfiI_PLK1, pGEM-T_SfiI_PLK1-PBD and pGEM-T_SfiI_PLK1-KD, respectively were excised with SfiI and cloned into pRTS-1. Plasmids with right orientation of the insert were confirmed by restriction digestion (Figure 45).

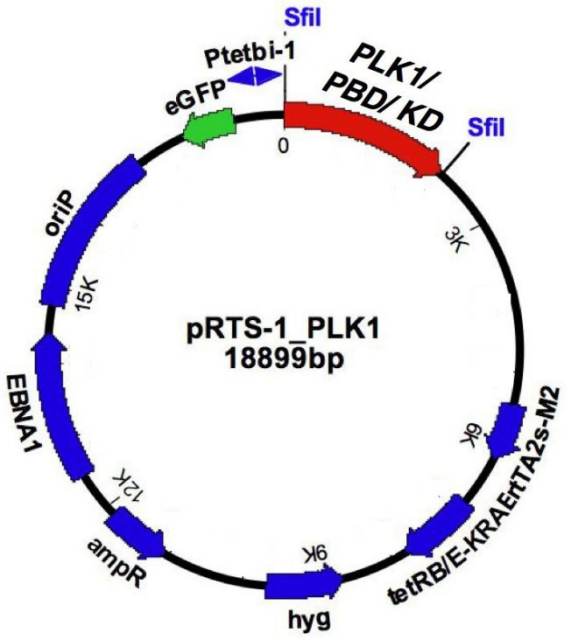

Figure 45: Subcloning of *PLK1*, *PBD* and *KD* in inducible mammalian expression vector pRTS-1. *PLK1*, *PBD* and *KD* fragments excised from pGEM-T_SfiI_PLK1, pGEM-T_SfiI_PBD, pGEM-T_SfiI_KD respectively with *Sfi*I flanking ends were ligated to pRTS-1 plasmid at *Sfi*I RE sites and clones were verified for proper orientation of inserted fragment.

5.2.2.4 Bacterial Culture and storage conditions

The *E. coli* cultures were routinely grown at 37ºC on Luria-Bertani (LB) agar or in LB broth, containing ampicillin (100 µg/ml) or kanamycine (30 µg/ml)

For long-term storage, a fresh overnight culture was prepared: Bacteria were streaked on to agar plate of the respective selective medium, and incubated overnight at 37ºC. Several colonies were transferred separately in to 10 ml falcon tubes containing 2 ml liquid media, and incubated for 14 - 16 h at 37ºC with shaking at 180-200 rpm. Then 800 µl of the bacterial culture were transferred into a storage tube and sterile glycerol was added to a final concentration of 20%. The vials were snap-frozen in liquid nitrogen, and stored at -80ºC until use. To recover a bacterial culture, a small proportion of the frozen glycerol stock was streaked onto an LB agar plate, containing the appropriate antibiotic. The plate was incubated overnight at 37ºC. These colonies were used as a working stock. The plates were sealed with parafilm and stored at 4ºC for up to four weeks. Alternatively, a small portion of the frozen glycerol stocks was transferred into 2 ml of the LB broth containing the appropriate antibiotic, and incubated overnight at 37ºC with shaking at 180-200 rpm.

5.2.2.5 Agarose gel electrophoresis

The gels were prepared by melting 1% (w/v) agarose in 1 x TBE buffer and adding 25 µl/l ethidium bromide. Each DNA sample was mixed with 1/6 volume of 6 x DNA loading buffer. Gels were run in 1X TBE buffer at a constant voltage of 100 V, imaged under UV-light and digitized for documentation.

5.2.2.6 Mini-preparation of plasmid DNA

For mini-preparation of plasmid DNA from bacteria, a QIAprep Spin Miniprep kit from Qiagen was used. For isolation of ~10 µg of Plasmid DNA, 2 ml overnight culture of *E. coli* DH5α in LB medium was prepared. Plasmid DNA was isolated according to the manufacturer's recommendations. DNA was eluted in 30 µl of the elution buffer and stored at -20°C.

5.2.2.7 Large-Scale preparation of plasmid DNA

For the large-scale plasmid isolation a QIAGEN Plasmid Midi and Maxi Kits were used. Plasmid DNA was isolated according to the supplier's manual, 200 ml of *E. coli* DH5α overnight culture was used for each experiment.

5.2.2.8 Measurement of DNA concentration

Concentration of DNA was measured using a UV-Spectrophotometer (Pharmacia Biotech) at the wavelength of 260 nm. The amount of DNA was calculated based on the optical density (OD) value ($OD_{260} = 1$ corresponds to 50 µg/ml double-stranded DNA).

5.2.2.9 Enzymatic manipulation and analysis of DNA

Digestion of DNA with restriction endonucleases

Digestion of the DNA with restriction endonucleases was performed according to the recommendations of the manufacturer. For the digestion of 5-10 µg of DNA, 10 U of enzyme was added with an appropriate buffer to the DNA in a final volume of 15 µl.

5.2.2.10 Vector dephosphorylation

To avoid self-ligation of restriction digested vector DNA, 5'-overhanging phosphate groups were cleaved off by adding 1 unit calf intestine alkaline phosphatase per 1 µg DNA and incubating for 1 h at 37°C.

5.2.2.11 DNA ligation

For the ligation of cDNA or PCR amplified DNA fragments into a plasmid vector T4 DNA ligase (New England Biolabs) was used. For ligation, 50-100 ng of the digested plasmid DNA was used. The amount of insert was calculated according to a molar ratio 3:1 (Insert:Vector). The reaction was incubated overnight at 16°C.

5.2.2.12 Polymerase chain reaction (PCR)

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. For amplification of DNA fragments by PCR, 50 µl reaction mixtures containing 10-50 ng plasmid DNA template, 10 pmol of forward primer, 10 pmol of reverse primer, 100 nmol dNTPs and 2.5 units Taq DNA polymerase in 1 x reaction buffer were prepared on ice. The PCR was carried out after an initial denaturation step of 30 sec at 95°C with 30 cycles comprising the following steps: 1) denaturation for 30 sec at 95° C, 2) annealing for 30 sec at 55°C (adjusted for each primer) and 3) elongation for 1.5 min per kb of fragment-length at 68°C. The final elongation step was extended by 10 min at 72°C.

5.2.2.13 Purification of PCR-amplified DNA fragments

PCR amplified DNA was either purified using the QIAquick PCR Purification Kit according to instructions or from 1% agarose gels with the QIAquick Gel Extraction Kit.

5.2.2.14 Transformation of *E. coli*

Competent *E.coli* DH5α were purchased from Invitrogen. Competent cells were thawed on ice and mixed gently. For each transformation, 40 µl of competent cells were transferred into a microcentrifuge tube and 20-50 ng of plasmid DNA or 1-3 µl of ligation mixture was

added, swirled gently and incubated on ice for 30 min. Tube with bacteria were heat-pulsed in a 42°C water bath for 45 to 90 sec. Then the tube was incubated on ice for 2 min and 900 µl of the LB medium pre warmed to 37°C was added and incubated at 37°C for 1 h with shaking at 180-200 rpm. 100 µl of transformation mixture was plated on the LB agar plates with appropriate antibiotic using a sterile spreader and incubated overnight at 37°C.

5.2.3 Immunoblotting

5.2.3.1 Preparation of SDS PAGE gels

According to the apparent molecular weight of the proteins in question, gels containing different concentrations of acrylamide were used. The composition of different gels is given in table below. Separation was performed at 180 V until the methylene blue front had left the gels ends.

Stacking gel	Acrylamide (40%) / mL	Tris/Cl pH7.0/ml	H2O /ml	SDS (10%) /µl	TEMED /µl	APS /µl
4%	0.375	0.380	2.185	30.0	3.0	30.0

Running gel	Acrylamide (40%) / mL	Tris/Cl pH7.0/ml	H2O /ml	SDS (10%) /µl	TEMED/ µl	APS /µl
8%	2.0	2.5	5.3	100	8	100
10%	2.5	2.5	4.7	100	4	100
12%	3.0	2.5	4.3	100	4	100
14%	3.5	2.5	3.7	100	4	100
16%	4.0	2.5	3.3	100	4	100

5.2.3.2 Composition of polyacrylamide gels

For one-dimensional gel electrophoresis acrylamide gels with the above given volumes of stock solutions were prepared. 10% aqueous APS was prepared freshly and the acrylamide stock solution contained acrylamide/ bisacrylamide in a ratio of 29:1.

5.2.3.3 Sample preparation for SDS PAGE

Cells were harvested at 300 *g* for 5 min and washed twice in an appropriate volume of 1X PBS. Lysis buffer was added and incubated for 30 min on ice. Debris was spun down at 16000 *g* for 15 min and the supernatant was collected as sample.

5.2.3.4 Determination of protein concentration

Protein concentration was determined using the bicinchonic assay kit by Pierce essentially according to the manufacture's protocol. 2 µl of sample was diluted in 200 µl of BCA solution in an ELISA plate (flat bottom) and incubated at room temperature for 30 min in the dark. Absorbance was measured using an ELISA reader at 620 nm. BSA was used as standard and the protein concentration was calculated on the basis of the derived standard curve.

5.2.3.5 Electrophoresis and immunoblotting of proteins

Proteins were separated under reducing conditions for 1 h at 180 V in 10-14% gels depending on the molecular weight of the respective protein and then blotted for 1 h at 1 mA/cm^2 onto the nitrocellulose membranes (Millipore) using blotting buffer and BioRad semi dry blotting apparatus (Transblot SD cell). Membranes were stained with Ponceau Red to verify homogenous protein transfer and destained in H_2O.

Membranes were blocked for 1 h in blocking solution and then incubated overnight with primary antibody. Excess primary antibody was removed, washed thrice, each for 5 min in 1X PBST and incubated with the peroxidase coupled secondary antibody for 1 h at room temperature. Excess antibody was washed off twice for 7 min each with 1X PBST and bound antibody was detected by enhanced chemiluminescence detection method (ECL kit, Pierce). Blots were sometimes stripped and probed with other antibodies.

5.2.3.6 Stripping of nitrocelulose membrane blots

Membranes were washed with 1X PBST for 5-10 min. Then about 50 ml of stripping solution was added and incubated in a shaking waterbath at 50°C for 30 min. The blots were then washed with 1X PBST and incubated with 7% blocking buffer in 1X PBST.

5.2.4 Immunofluorescence microscopy

Cells were grown on coverslips and fixed in 4% formaldehyde for 30 min, and permeabilized with 0.2% Triton X-100 + PBS for 10 min at room temperature. Afterwards cells were washed and incubated for 30 min at room temperature in blocking solution (PBS+ 1% bovine serum albumin). Mouse monoclonal anti-β-tubulin (1:1000; Sigma) antibody was used for staining □-tubulin. Cells were incubated with primary antibody for 2 h at room temperature in a humidified chamber, followed by three washes in PBS. Subsequently, cells were incubated with Alexa Fluor 594–conjugated chicken anti–mouse IgG [Invitrogen] secondary antibody for 1 h at room temperature. For staining DNA, cells were incubated in PBS + 4,6-diamidino-2-phenylindole (DAPI; 1mg/ml) for 5 min. Cells were washed and mounted in fluorescence mounting medium (Dako). For sub-cellular localization pattern analysis, cells were inspected with a microscope (Axiovert 200; Carl Zeiss, Inc.) equipped with a 63x/1.4 and 100x objective lens (Plan-Apochromat; Carl Zeiss, Inc.) and a digital camera (ORCA ER; Hamamatsu Photonics). Images were acquired by Openlab software (Improvision) and vertical slices (0.2-µm separation) were deconvoluted with Openlab 5.0.2 nearest–neighbor deconvolution algorithm on Mac OSX 10.4 (Apple Inc.).

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7 APPENDIX

7.1 ABBREVIATIONS

AIF	apoptosis inducing factor
APS	Ammonium persulphate
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CAPS	3-cyclohexylamino-1-propanesulfonic acid
CARD	Caspase activation and recruitment domain
Caspase	cysteinyl aspartate-specific protease
CO_2	Carbon dioxide
cyt c	Cytochrome c
dATP	deoxyadenosin triphosphate
DED	Death effector domain
DISC	Death inducing signaling complex
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
e.g	For example (latin:'exempli gratia')
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	fluorescence activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
g	Gravity
GM	Growth medium
h	Hour
H_2O	Water
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HRP	Horse radish peroxidase
i.e.	That is (latin: 'id est')
IF	Immunofluorescence
Ig	Immunoglobulin
KDa	Kilo Dalton
KD	Kinase domaion of PLK1
l	Liter
mA	Milliampere
min	Minute
miRNA	MicroRNA
ml	Milliliter
mm	millimeter
mM	Millimolar
NCCD	The Nomenclature Committee on Cell Death
nM	Nanomolar
PBS	Phosphate buffered saline
PBD	Polo Box Domain of PLK1
PCD	Programmed cell death
PLK1	Polo Like Kinase 1
PS	Phosphotidyl serine
pH	Negative decadic logarithm of the hydrogen ion concentration (latin: 'potentia hydrogenii')
PI	Propidium Iodid
PVDF	Polyvinylidene fluoride
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-Tetramethylethylenediamine
UV	Ultraviolet
V	Volts

w/v	weight/volume
µF	Microfarad
µl	Microliter
µM	Micromolar

7.2 Erklärung

Hiermit erkläre ich, Cindrilla Chumduri, geb. am 11.10.1980 in Davangere-KS, Indien, die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben und alle verwendeten Hilfsmittel und Inhalte aus anderen Quellen als solche kenntlich gemacht zu haben. Desweiteren versichere ich, dass die vorliegende Arbeit nie in dieser oder anderer Form Gegenstand eines früheren Promotionsverfahrens war. Die dem angestrebten Promotionsverfahren an der Mathematischen-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin zugrunde liegende Promotionsordnung ist mir bekannt.

Berlin, im August 2009 _____

Unterschrift

7.3 Publications

Cindrilla Chumduri, Bernhard Gillissen, Peter T. Daniel. Mitotic catastrophe is a failsafe mechanism in Burkitt lymphomas treated with mitotic spindle poisons. *(Manuscript in preparation)*

Cindrilla Chumduri, Bernhard Gillissen, Peter T. Daniel. PLK1 deregulation triggers mitotic catastrophe in a caspase dependent manner. *(Manuscript in preparation)*

Conferences and Presentations

THE CELL CYCLE, 17th – 21st May 2006, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA

Oral Presentation - Ways of cell death in Burkitt's lymphoma: Apoptotsis or Mitotic catastrophe. European Students Conference, 7th – 11th October 2007, Charité-Universitatmedizin Berlin, Germany

Poster presentation – **Cindrilla Chumduri**, Bernhard F. Gillissen, Antje Richter, Anja Richter, Peter T. Daniel. Ways of cell death in Burkitt's lymphoma: Apoptotsis or Mitotic catastrophe. European Workshop on Cell Death, 1st – 6th June 2008, Hauenstein, Germany

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